Supporting Information

Stereoselective Enzymatic Synthesis of Heteroatom-Substituted Cyclopropanes

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Supporting Information content:

I. Experimental Procedures

General Unless otherwise noted, all chemicals and reagents were obtained from commercial suppliers (Sigma-Aldrich, VWR, Alfa Aesar) and used without further purification. Silica gel chromatography was carried out using AMD Silica Gel 60, 230-400 mesh. Synthetic reactions were monitored using thin layer chromatography (Merck 60 gel plates) using a UV-lamp for visualization. ^{1}H and ^{13}C NMR spectra were recorded on a Varian Inova 300 MHz or 500 MHz, or Bruker Prodigy 400 MHz instrument, in $CDCl_3$ and are internally referenced to the residual solvent peak. Data for ^{1}H NMR are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, ddd = doublet of doublet of doublets), coupling constant (Hz), integration. High-resolution mass spectra were obtained at the California Institute of Technology Mass Spectrometry Facility.

Chromatography. Analytical high-performance liquid chromatography (HPLC) was carried out using an Agilent 1200 series instrument and an Agilent Eclipse XDB-C18 column (4.6 x 150 mm, 5 μm) with water and acetonitrile as the mobile phases. Exemplary HPLC runs for *N*-vinylphthalimide cyclopropanation are shown in Figure S7. For quantitative HPLC analysis of *N*-vinylphthalimide cyclopropanation reaction products, calibration curves using ethyl benzoate as internal standard were generated (Figure S8). The identity of the reaction products was confirmed by HPLC co-injections of reaction mixtures with chemically synthesized authentic products, and by NMR analysis of products isolated from reactions performed on preparative scale. Cyclopropanation product enantiomers were separated using chiral SFC with isopropanol and liquid CO_2 as the mobile phase, or chiral HPLC with isopropanol and hexane as the mobile phase. Product enantiomers were separated using Chiralpak IA, IC, or OJ-H columns (4.6 x 250 mm, 5 μm) from Chiral Technologies Inc.

Cloning and site-saturation mutagenesis. pET22b(+) with the pelB leader sequence removed was used as a cloning and expression vector for all constructs described in this study. Site-saturation libraries were generated employing the 22c-trick method[1]. The resulting PCR products were gel purified, digested with DpnI, repaired using the method of Gibson[2], and used to directly transform *E. coli* strain BL21(DE3).

Determination of P411 concentration. The concentration of P411 enzymes in whole cell experiments was determined from ferrous carbon monoxide binding difference spectra using the previously reported extinction coefficient for serine-ligated enzymes (ε = 103,000 M^{-1} cm^{-1})[3]. The concentration of purified P411 enzymes was determined by quantifying the amount of free hemin using the pyridine/hemochrome assay using the corresponding extinction coefficient (ε = 191,500 M^{-1} cm^{-1})[4].

Expression and small-scale *N*-vinylphthalimide cyclopropanation bioconversions using whole cells. *E. coli* BL21(DE3) cells transformed with plasmid encoding P411 variants were grown overnight in 3 mL Luria-Bertani medium with 0.1 mg/mL ampicillin (LB_{amp}) at 30 ºC and 250 rpm. Precultures were centrifuged (3000 g, 3 minutes), the supernatant was discarded, and cell pellets were resuspended in 3 mL fresh LB_{amp} and used to inoculate 47 mL of Hyperbroth medium (prepared from AthenaES© powder, 0.1 mg/mL ampicillin) in a 125 mL Erlenmeyer flask. Cultures were incubated at 37 ºC, 230 rpm for 2 h, typically reaching an OD_{600} = 1.5. Cultures were then cooled on ice (20 min) and induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and 1.0 mM 5-aminolevulinic acid (ALA) (final concentrations). Expression was conducted at 22 ºC, 130 rpm, for 16-18 h. Cultures were then centrifuged (3000 g, 5 min, 4 ºC) and the pellets were resuspended to OD_{600}= 30 in M9-N minimal medium (47.7 mM Na_2HPO_4, 22.0 mM KH_2PO_4, 8.6 mM NaCl, 2.0 mM $MgSO_4$, and 0.1 mM $CaCl_2$, pH 6.8). To determine P411 expression levels, aliquots of the cell suspension (4 mL) were lysed by sonication (Qsonica Q500 sonicator), and the lysate was cleared by centrifugation (13,000 g, 10 min, 4 ºC) and subjected to the carbon monoxide binding assay specified above.

Small-scale cyclopropanation reactions were set up in 2 mL crimp vials at 500 µL reaction volume. P411-expressing cells at OD_{600} = 30 in M9-N were degassed by sparging with argon in sealed 6 mL crimp vials for at least 30 minutes. Separately, a glucose solution (500 mM in M9-N) was degassed by sparging with argon for at least ten minutes. Following argon sparging, cells, glucose solution, and substrate solutions were transferred into an anaerobic chamber. The cells (440 or 465 µL) were added to 2 mL crimp vials, followed by glucose solution (10 µL), olefin (12.5 or 25 µL of a DMSO stock), and EDA (12.5 or 25 µL of an EtOH stock). Final concentrations were typically 5 to 30 mM olefin, 10 to 60 mM EDA, 10 mM glucose, and 5 or 10% cosolvent. The

vials were sealed, removed from the anaerobic chamber, and shaken at room temperature, 500 rpm for 16–20 h. The reactions were quenched by adding acetonitrile (1.5 mL) supplemented with internal standard (ethyl benzoate, to 0.5 mM final concentration). This mixture was transferred to a 2 mL microcentrifuge tube and centrifuged at 13,000 g for 10 minutes. The cleared supernatant was transferred to a vial and analyzed by HPLC.

Site-saturation library screening in 96-well plate format. Single colonies of *E. coli* cells transformed with site-saturation libraries were picked with sterile toothpicks and cultured in 96-well deep-well plates in LB_{amp} (300 μL/well) at 37 ºC, 250 rpm, overnight. In a fresh 96-well deep-well plate, hyperbroth medium (950 μL/well, 0.1 mg/mL ampicillin) was inoculated with the precultures (50 μL/well) and incubated at 37 ºC, 250 rpm, for 2.5 h. The plates were cooled on ice for 20 minutes and then induced with 0.5 mM IPTG and 1.0 mM ALA (final concentrations). Expression was conducted at 20 ºC, 200 rpm for 20 h. The cells were pelleted (3,000 g, 5 min), the supernatant discarded, and the 96-well plates were transferred to an anaerobic chamber. In the anaerobic chamber, cell pellets were resuspended in argon-sparged reaction buffer (20 mM glucose in M9-N, 470 μL/well), and stocks of olefin (15 μL/well, in DMSO) and EDA (15 μL/well, in EtOH) were added. Substrate concentration varied in the course of directed evolution between 10 and 30 mM for both EDA and *N*-vinylphthalimide. In later screening rounds, cells were resuspended in 440 μL M9-N/glucose buffer followed by addition of 30 μL of each substrate stock, in order to achieve higher co-solvent concentrations and thus aid with *N*-vinylphthalimide solubility at higher substrate concentrations. The plates were sealed with aluminum foil, removed from the anaerobic chamber, and shaken at 50 rpm. After overnight incubation, the seal was removed and acetonitrile (500 μL/well) supplemented with internal standard (ethyl benzoate, to 0.5 mM final concentration) was added. The plates were resealed, briefly mixed by vortexing, and incubated for 30 min to 1 h at room temperature. The plates were then centrifuged (5,000 g, 10 min), and the cleared supernatant was filtered through an AcroPrep 96-well filter plate (0.2 μm cutoff) into a shallow-well plate for HPLC analysis.

Protein purification. *E. coli* BL21(DE3) cells freshly transformed with plasmid encoding P411 variants were grown overnight in 25 mL LB_{amp} (30 ºC, 250 rpm). Hyperbroth medium (500 mL, 0.1 mg/mL ampicillin) in a 2.8 L flask was inoculated with 20 mL of the preculture and incubated

at 37 ºC, 230 rpm for 2 h (to OD_{600} ca. 1.5). Cultures were then cooled in an ice-water bath for 20 min and induced with 0.5 mM IPTG and 1.0 mM ALA (final concentrations). Expression was conducted at 22 ºC, 140 rpm, for 16-20 h. Cultures were then centrifuged (5,000 g, 10 min, 4 ºC) and the cell pellets were frozen at –20 ºC. For protein purification, frozen cells from two such cultures were resuspended in buffer A (25 mM tris, 20 mM imidazole, 100 mM NaCl, pH 7.5, 4 mL/g of cell wet weight), loaded with hemin (1 mg/gram wet cell weight) and lysed by sonication. To pellet insoluble material, lysates were centrifuged (20,000 g, 20 min, 4 ºC). P411 proteins were purified from the lysate using a nickel NTA column (1 mL HisTrap HP, GE Healthcare, Piscataway, NJ) using an AKTAxpress purifier FPLC system (GE healthcare). P411 enzymes were eluted on a linear gradient from 100% buffer A to 100% buffer B (25 mM tris, 300 mM imidazole, 100 mM NaCl, pH 7.5) over 10 column volumes. Fractions containing eluted protein were pooled and subjected to three rounds of buffer exchange to storage buffer (25 mM Tris-HCl, 25 mM NaCl, pH 7.5) using centrifugal spin filters (10 kDa molecular weight cut-off, Amicon Ultra, Merck Millipore). Subsequently, the concentrated protein was aliquoted, flash-frozen on powdered dry ice, and stored at –80 ºC. Protein concentrations were determined via the pyridine/hemochrome assay specified above.

Cyclopropanation reactions using purified protein. Portions of M9-N buffer (290 μL) and NADPH (40 μL, 50 mM in M9-N), or multiples thereof, were combined in a 6 mL crimp vial and degassed by sparging with argon for at least 30 minutes. Purified protein solutions were adjusted to 25 μM in M9-N. After degassing was complete, the M9-N/NADPH solution and purified protein stocks were brought into the anaerobic chamber. Reactions were set up on a 400 μL scale in 2 mL crimp vials: first, 330 μL of the M9-N/NADPH solution were added per vial, followed by 40 μL of purified protein solution. Next, 20 μL of *N*-vinylphthalimide stock solution (100 mM in DMSO) were added, followed by 10 μL of EDA stock solution (200 mM in EtOH). Final concentrations were typically 5 mM *N*-vinylphthalimide, 5 mM EDA, 5 mM NADPH, and 2.5 μM P411, with 7.5% co-solvent. The vials were sealed, removed from the anaerobic chamber, and shaken at room temperature, 40 rpm for 1 to 6 h. The reactions were quenched by adding acetonitrile (400 μL) supplemented with internal standard (ethyl benzoate, to 0.5 mM final concentration). This mixture was transferred to a microcentrifuge tube and centrifuged at 14,000 rpm for 10 minutes. The supernatant was transferred to a vial and analyzed by HPLC.

Determination of initial rates. (i) With purified protein: Reactions were set up as described above (*Cyclopropanation reactions using purified protein*). Reactions were quenched in 1-minute intervals (starting 2 min after the start of the reaction) by addition of 400 μL acetonitrile containing internal standard, followed by vigorous mixing. The samples were then worked up and analyzed as described above. **(ii)** With whole cells: Reactions were set up as described above (*Expression and small-scale N-vinylphthalimide cyclopropanation bioconversions using whole cells*). Reactions were quenched in 1-minute intervals (starting 2 min after the start of the reaction) by addition of 500 μL acetonitrile, followed by vigorous mixing. The vials were removed from the anaerobic chamber and 1 mL of acetonitrile supplemented with internal standard (ethyl benzoate, to 0.5 mM final concentration) was added. Further sample work-up and HPLC analysis was performed as described above.

Preparative scale reactions. *E. coli* BL21(DE3) cells freshly transformed with plasmid encoding P411 variants were grown overnight in 25 mL LB_{amp} (30 ºC, 250 rpm). Hyperbroth medium (500 mL, 0.1 mg/mL ampicillin) in a 2.8 L flask was inoculated with 20 mL of the preculture and incubated at 37 ºC, 230 rpm for 2 h (to OD_{600} ca. 1.5). Cultures were then cooled in an ice-water bath for 20 min and induced with 0.5 mM IPTG and 1.0 mM ALA (final concentrations). Expression was conducted at 22 ºC, 140 rpm, for 16-20 h. Cultures were then centrifuged (5,000 g, 5 min, 4 ºC) and the cell pellets were resuspended to OD_{600} = 50 in M9-N buffer supplemented with 20 mM glucose (typically yielding ca. 110 mL of cell suspension). Aliquots of the cell suspension (4 mL) were used to determine the P411 expression level after cell lysis by sonication. Aliquots of the cell suspension (23 mL) were then transferred to 50 mL Erlenmeyer flasks and degassed by sparging with argon for at least 30 minutes. The reaction flasks were then transferred into an anaerobic chamber and olefin stock solution (1 mL, in DMSO) and EDA (1 mL, in EtOH) were added. Final concentrations were typically 5 to 20 mM olefin and 10 to 40 mM EDA, with 8% co-solvent. The flasks were sealed with parafilm, removed from the anaerobic chamber, and shaken at room temperature, 100 rpm for 18 h. The reactions were quenched by adding acetonitrile (25 mL) and then centrifuged (4,000 g, 10 min). The supernatant was concentrated *in vacuo* to remove acetonitrile and extracted with EtOAc (3 x 25 mL). The organic layers were washed with brine (20 mL), dried over $MgSO_4$, filtered, concentrated, and purified by silica gel chromatography.

Assignment of absolute configuration. To assign the absolute configuration of the *trans*-configured *N*-vinylphthalimide cyclopropanation product (ethyl *trans*-2-(1,3-dioxoisoindolin-2-yl)cyclopropane-1-carboxylate, **3b**), purified product from preparative-scale enzymatic reactions with P411-VAC$_{trans}$ was subjected to crystallization and X-ray structure determination (Table S1). Low-temperature diffraction data (ϕ-and ω-scans) were collected on a Bruker AXS D8 VENTURE KAPPA diffractometer coupled to a PHOTON 100 CMOS detector with Cu K_α radiation (λ = 1.54178 Å) from an IμS micro-source. The structure was solved by direct methods using SHELXS and refined against F^2 on all data by full-matrix least squares with SHELXL-2016[5] using established refinement techniques. All non-hydrogen atoms were refined anisotropically. All hydrogen atoms were included into the model at geometrically calculated positions and refined using a riding model. The isotropic displacement parameters of all hydrogen atoms were fixed to 1.2 times the *U* value of the atoms they are linked to (1.5 times for methyl groups). Ethyl *trans*-2-(1,3-dioxoisoindolin-2-yl)cyclopropane-1-carboxylate **3b** crystallizes in the orthorhombic space group $P2_12_12_1$ with one molecule in the asymmetric unit. The absolute configuration was assigned as *trans*-(1*S*, 2*S*) (Figure S1). The absolute configurations of other cyclopropanes formed by P411-VAC$_{trans}$ were assigned by analogy. Crystallographic coordinates and structure factors for ethyl *trans*-2-(1,3-dioxoisoindolin-2-yl)cyclopropane-1-carboxylate **3b** have been deposited with the Cambridge Crystallographic Data Centre (https:// www.ccdc.cam.ac.uk/) under reference number 1815565.

Attempts to crystallize the *cis*-configured *N*-vinylphthalimide cyclopropanation product were unsuccessful; furthermore, attempts to crystallize alternative *cis*-configured products derived from P411-VAC$_{cis}$ (Table 1) were unsuccessful as well. We therefore determined the selectivity of P411-VAC$_{cis}$ in the cyclopropanation reaction of styrene with EDA (Figure S2), as previously described[6]. To this end, whole-cell reactions with *E. coli* cells expressing P411-VAC$_{cis}$ at OD_{600}=30 were performed with 10 mM styrene and 10 mM EDA, following the protocol described above (*Expression and small-scale N-vinylphthalimide cyclopropanation bioconversions using whole cells*). Reactions were extracted with cyclohexane and analyzed by GC using a Cyclosil-B column (30m x 0.32 mm x 0.25 µm), oven temperature = 100 °C for 5 min, 1 °C per min to 135°C, 135 °C for 10 min, 10 °C per min to 200 °C, 200 °C for 5 min[6]. Using a racemic product standard for comparison and a previously developed assay[6], the major styrene cyclopropanation product

isomer derived with P411-VAC$_{cis}$ was thereby assigned as (1*S*, 2*R*), and we assigned the absolute configuration of other cyclopropanes formed by P411-VAC$_{cis}$ (or its single point mutation derivatives) by analogy. Of note, we also included P411-VAC$_{trans}$ in this experiment; the major styrene cyclopropanation product isomer obtained with this variant is the (1*S*, 2*S*) isomer, in agreement with the absolute configuration obtained by crystal structure determination (Figures S1, S2).

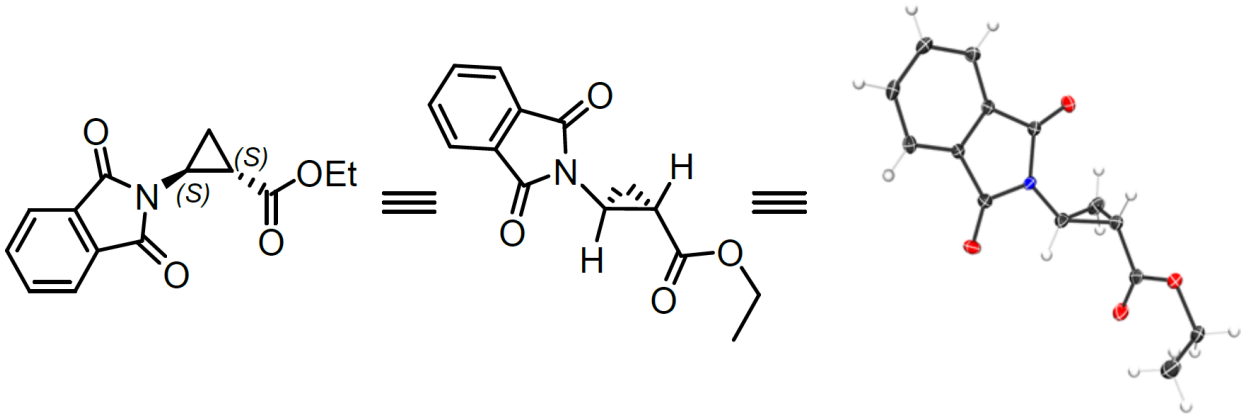


Figure S1: **Crystal structure of ethyl *trans*-2-(1,3-dioxoisoindolin-2-yl)cyclopropane-1-carboxylate, 3b.** The absolute configuration of **3b** was assigned as (1*S*, 2*S*).

Table S1. Crystal data and structure refinement for ethyl (1S, 2S)-2-(1,3-dioxoisoindolin-2-yl) cyclopropane-1-carboxylate (**3b**).

Empirical formula	$C_{14}H_{13}NO_4$
Formula weight	259.25
Temperature	100 K
Wavelength	1.54178 Å
Crystal system	Orthorhombic
Space group	$P2_12_12_1$
Unit cell dimensions	a = 6.6275(5) Å, α = 90°
	b = 8.0167(7) Å, β = 90°
	c = 22.6419(19) Å, γ = 90°
Volume	1202.98(17) $Å^3$
Z	4
Density (calculated)	1.431 Mg/m^3
Absorption coefficient	0.883 mm^{-1}
F(000)	544
Crystal size	0.350 x 0.300 x 0.250 mm^3
Theta range for data collection	3.905 to 74.466°
Index ranges	-8<=h<=8, -10<=k<=10, -28<=l<=25
Reflections collected	13445
Independent reflections	2442 [R(int) = 0.0319]
Completeness to theta = 67.679°	99.8 %
Absorption correction	Semi-empirical from equivalents
Max. and min. transmission	0.7538 and 0.6780
Refinement method	Full-matrix least-squares on F^2
Data / restraints / parameters	2442 / 0 / 173
Goodness-of-fit on F^2	1.154
Final R indices [I>2sigma(I)]	R1 = 0.0334, wR2 = 0.0889
R indices (all data)	R1 = 0.0336, wR2 = 0.0892
Absolute structure parameter	0.07(5)
Extinction coefficient	n/a
Largest diff. peak and hole	0.218 and -0.329 $e.Å^{-3}$

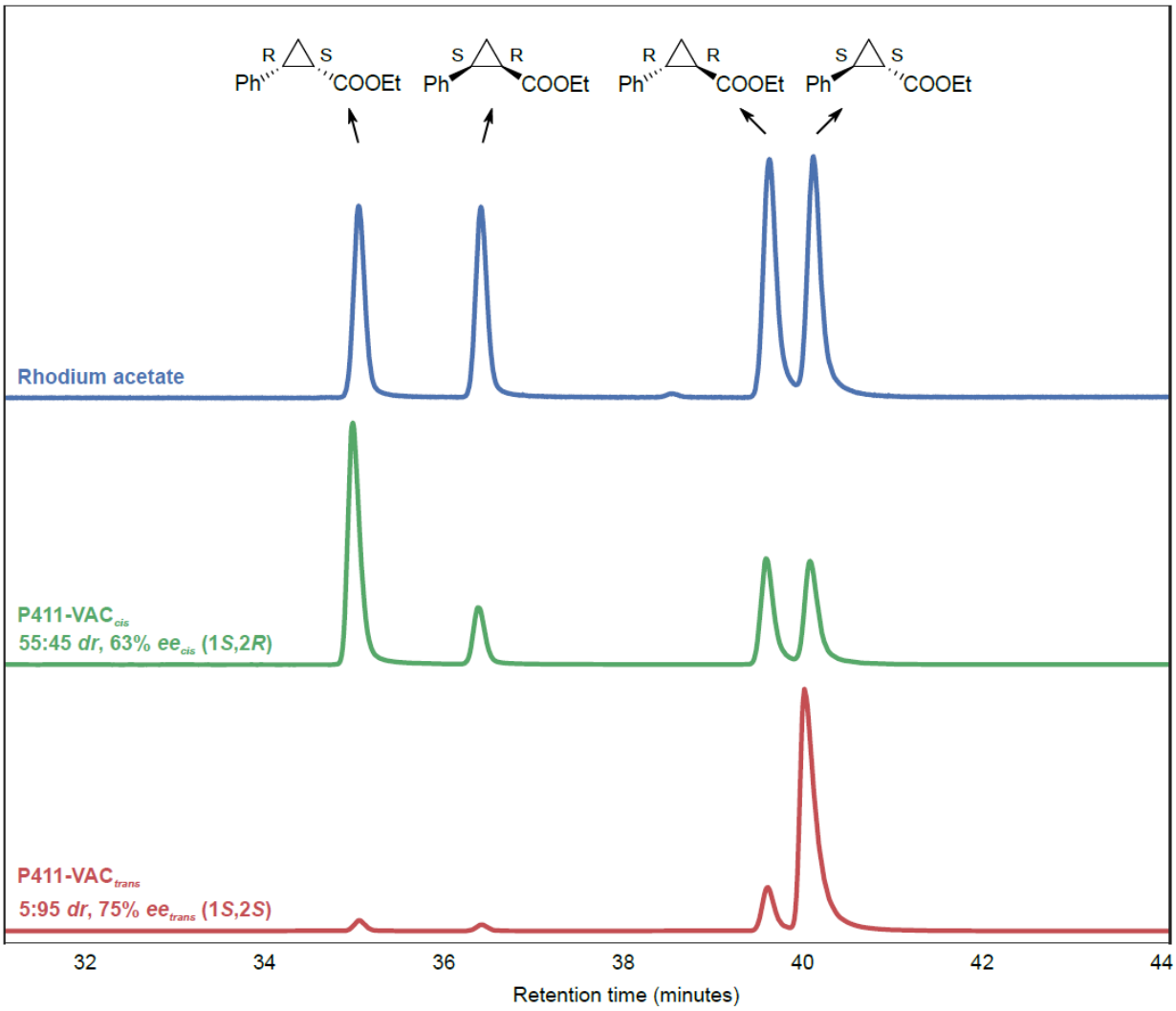
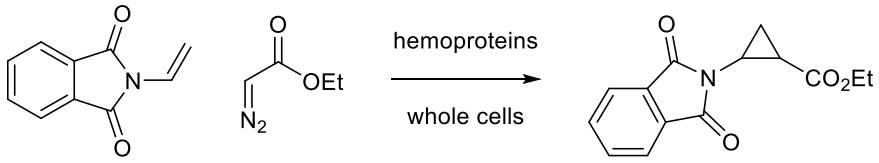


Figure S2: Assigning absolute configuration of styrene cyclopropanation products obtained with P411-VAC$_{cis}$ and P411-VAC$_{trans}$. We tested P411-VAC$_{cis}$ and P411-VAC$_{trans}$ for the cyclopropanation of styrene with EDA (see section *Assignment of N-vinylphthalimide cyclopropanation product absolute configuration* above for experimental details). For P411-VAC$_{cis}$, this delivered the (1*S*, 2*R*) isomer as major product, and the absolute configuration of other P411-VAC$_{cis}$ cyclopropanation products was assigned by analogy. The major styrene cyclopropanation product isomer obtained with P411-VAC$_{trans}$ was assigned as (1*S*, 2*S*), in agreement with the absolute configuration of the *N*-vinylphthalimide cyclopropanation product obtained with that variant as determined by crystallography (*vide supra*).

II. Supplementary Tables

Table S2. Hemoprotein variant screening for *N*-vinylphthalimide cyclopropanation.



Hemoprotein variant[a]	**Description, mutations, references**	**Yield [%]**	***dr* (*cis*:*trans*)**
Hemin[b]	Free hemin in buffer, no protein	1.5	28:72
E. coli BL21(DE3)	No protein overexpression	N.D.	-
$P450_{BM3}$	Wild-type $P450_{BM3}$	0.2	37:63
$P450_{BM3}$ T268A	$P450_{BM3}$ with mutation T268A[6]	0.2	39:61
$P450_{BM3}$ T268A C400S	$P450_{BM3}$ with mutations T268A and C400S[7]	0.5	31:69
$P450_{BM3}$ T268A C400H	$P450_{BM3}$ with mutations T268A and C400H	0.5	35:65
$P450_{BM3}$-CIS	$P450_{BM3}$ with mutations V78A F87V P142S T175I A184V S226R H236Q E252G T268A A290V L353V I366V E442K[6]	0.3	56:54
$P411_{BM3}$-CIS[7]	$P450_{BM3}$-CIS with C400S mutation	2.0	88:12
$P450_{BM3}$-CIS C400H	$P450_{BM3}$-CIS with C400H mutation	1.6	74:26
H2-5-F10	$P450_{BM3}$ with mutations L75A V78A F87V P142S T175I A184V S226R H236Q E252G I263A T268A A290V L535V I366V L437A E442K[6]	0.2	45:55

H2-5-F10 C400S	H2-5-F10 with mutation C400S	1.1	56:54
H2-5-F10 C400H	H2-5-F10 with mutation C400H	0.5	35:65
HStar H92N H100N	$P450_{BM3}$ with mutations V78M H92N H100N L181V T268A C400H L437W[8, 9]	0.5	27:73
P450-Cam	P450cam from *Pseudomonas putida*[10]	0.5	38:62
Cyp119	Cyp119 from *Sulfolobus acidocaldarius*[11]	0.9	44:56
Cyt *c Rma* TDE	Cytochrome *c* from *Rhodothermus marinus* evolved for carbene Si–H insertion[12]	1.2	28:72
Ape protoglobin	Protoglobin from *Aeropyrum pernix*[13]	0.6	52:48

[a] Reactions were performed in 96-well plates with whole *E. coli* cells expressing the respective hemoprotein variants, at 20 mM substrate loading. Results are the average of duplicate reactions. N.D. = not detected. [b] Reaction with hemin: in the anaerobic chamber, 40 μL of hemin solution (1 mM in DMSO) were added to 310 μL of argon-sparged M9-N buffer, followed by 10 μL of sodium dithionite as reductant (400 mM in M9-N buffer), *N*-vinylphthalimide (20 μL, 400 mM in DMSO) and EDA (20 μL, 400 mM in DMSO). Final concentrations were 100 μM hemin, 10 mM sodium dithionite, 20 mM *N*-vinylphthalimide and 20 mM EDA with 20% co-solvent (DMSO). The observed yield corresponds to 3 TTN. Note that P450 variants in 96-well plates typically express at ~0.5 μM, *i.e*. 200-fold lower than the hemin concentration tested here.

Table S3a. Summary of directed evolution for *cis*-selective *N*-vinylphthalimide cyclopropanation.

Round	**Parent variant**	**Site-saturation libraries evaluated**[a]	**Screening substrates**	**Mutations identified**
1	$P411_{BM3}$-CIS	V87X, L181X, I263X, T268X, L437X, T438X	*N*-vinylphthalimide EDA	V87T, I263A/F/G, L437F/Y, T438C/Q
2	$P411_{BM3}$-CIS	Recombination library of mutations[b]: V87T, I263A/F/G, L437F/Y, T438C/Q	*N*-vinylphthalimide EDA	L437F T438Q
3	$P411_{BM3}$-CIS L437F T438Q	A328X, Q387X, I263X, A264X, L75X	*N*-vinylphthalimide EDA	L75Y
4	$P411_{BM3}$-CIS L437F T438Q L75Y	V87X, L181X	*N*-vinylphthalimide EDA	L181I
Final variant: **P411-VAC$_{cis}$** = $P411_{BM3}$-CIS L75Y L181I L437F T438Q				

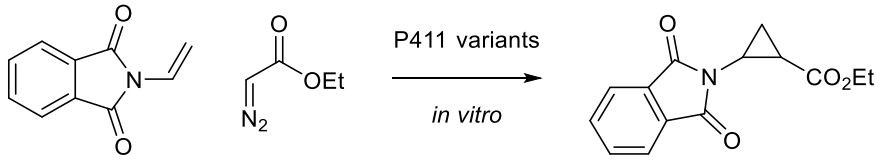
[a] Some residues were saturated twice, in different parent variants. [b] The corresponding wild-type residues at each position were included in the recombination library.

Table S3b. Summary of directed evolution for *trans*-selective *N*-vinylphthalimide cyclopropanation.

Round	**Parent variant**	**Site-saturation libraries evaluated**[a]	**Screening substrates**	**Mutations identified**
1	$P411_{BM3}$-CIS	V87X, L181X, I263X, T268X, L437X, T438X	*N*-vinylphthalimide EDA	V87T, I263A/F/G, L437F/Y, T438C/Q
2	$P411_{BM3}$-CIS	Recombination library of mutations[b]: V87T, I263A/F/G, L437F/Y, T438C/Q	*N*-vinylphthalimide EDA	I263G L437F
3	$P411_{BM3}$-CIS I263G L437F	A328X, T438X, L75X, V87X	*N*-vinylphthalimide EDA	V87L
4	$P411_{BM3}$-CIS I263G L437F V87L	L181X	*N*-vinylphthalimide EDA	L181R
Final variant: **P411-VAC*trans*** = $P411_{BM3}$-CIS V87L L181R I263G L437F				

[a] Some residues were saturated twice, in different parent variants. [b] The corresponding wild-type residues at each position were included in the recombination library.

Table S4. *N*-vinylphthalimide cyclopropanation reactions performed with purified P411 variants.[a]



Catalyst	Yield [%]	TTN	*dr* [*cis*:*trans*]	*ee* [%]
$P411_{BM3}$-CIS	6.1	120	64:36	-9
P411-VAC_{cis}	31	620	91:9	93
P411-VAC_{trans}	26	510	8:92	84

[a] Reactions were performed with 2.5 μM P411, 5 mM NADPH, 5 mM *N*-vinylphthalimide, and 5 mM EDA; results are the average of duplicate reactions.

Table S5. Library of P411-VAC_{cis} variants with single active site mutations at residues V87, I263, E267 and A328.

Position	Variants[a]
V87	F, C, T, I
I263	A, T, V
E267	V, N, Q
A328	N, T, S, Q

[a] These variants were identified in the course of directed evolution projects aimed at improving P411-VAC_{cis} for unactivated alkene cyclopropanation reactions[13].

III. Supplementary Figures

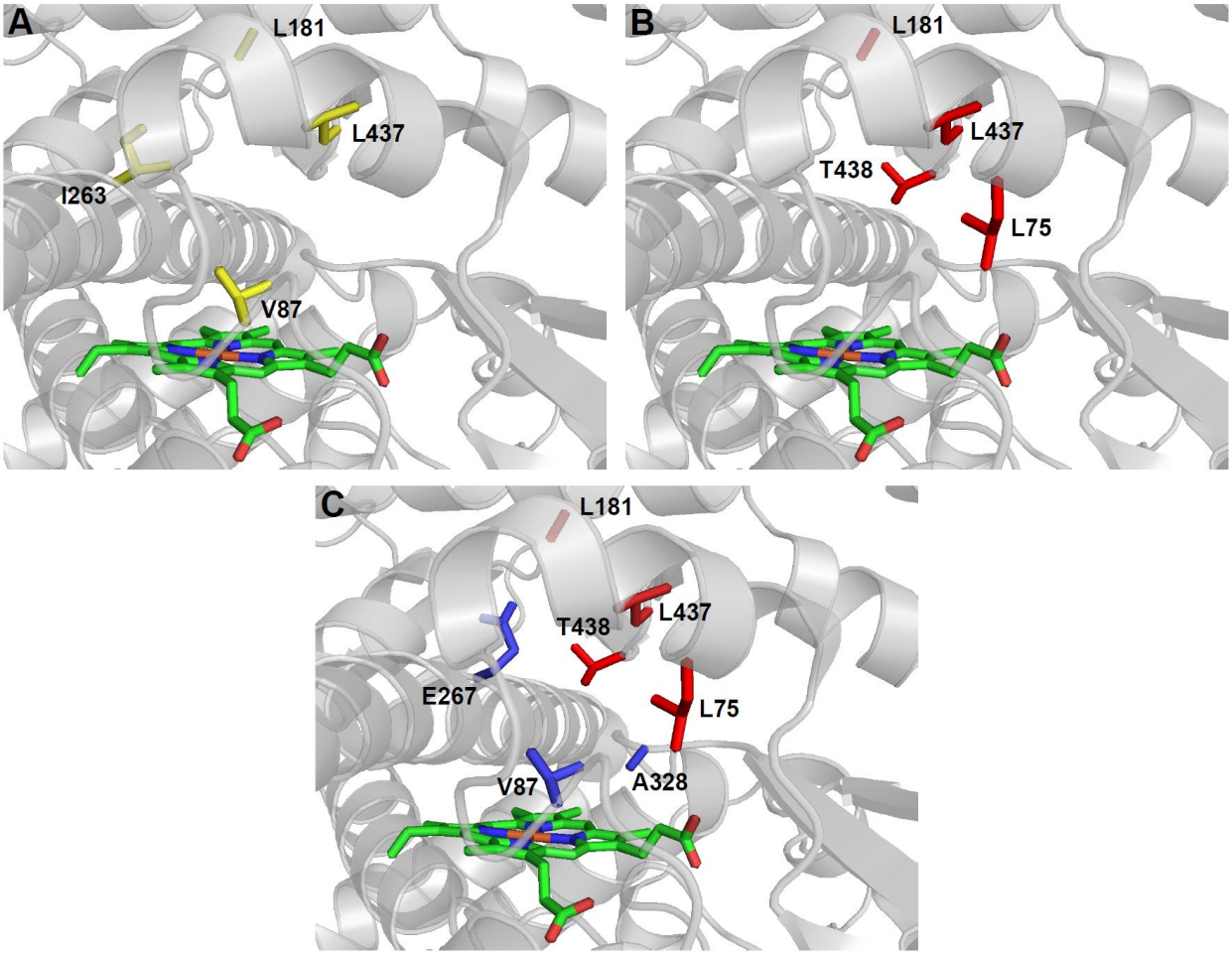


Figure S3: Position of active site mutations in P411-VAC*trans* and P411-VAC*cis* variants. Structures are based on the parent enzyme P411$_{BM3}$-CIS (pdb: 4h23)[7] and depict the active site with the heme cofactor. The sidechains of residues mutated in P411-VAC*trans* and P411-VAC*cis* are shown as colored sticks. All residues targeted for mutagenesis were chosen due to their proximity to the heme cofactor and because mutations at these positions were known to affect activity or selectivity of cytochrome P450-catalyzed cyclopropanation reactions.[6,7,9,13] (A) Position of altered residues (yellow) in variant P411-VAC*trans* (P411$_{BM3}$-CIS I263G L437F V87L L181R). (B) Position of altered residues (red) in variant P411-VAC*cis* (P411$_{BM3}$-CIS L437F T438Q L75Y L181I). (C) Positions of additional single-residue mutations (blue) in P411-VAC*cis* to enhance diastereo- and enantioselectivities across multiple substrates (see Table 1, Figure S6). Ongoing

structure determination efforts may provide further insight into the molecular basis of the catalytic activity and diastereoselectivity of P411-VAC$_{trans}$ and P411-VAC$_{cis}$.

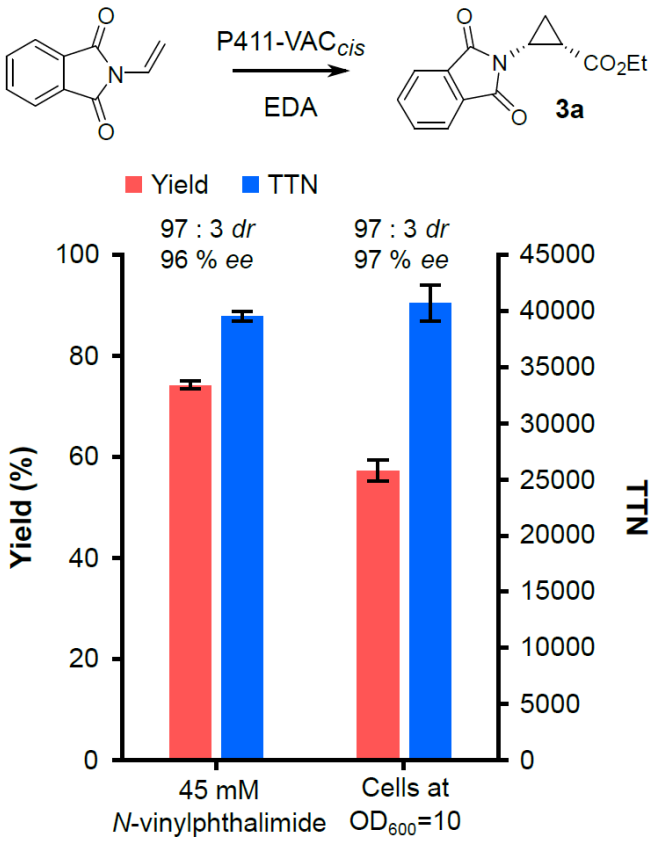


Figure S4: Maximizing TTN for P411-VAC$_{cis}$. To test for maximal TTN of the *cis*-selective variant P411-VAC$_{cis}$ two different reaction conditions were investigated. First, *N*-vinylphthalimide substrate loading was increased to 45 mM (compared to 30 mM as shown in Figure 1A) with 60 mM EDA, using whole cell catalysts at OD$_{600}$=30. Under these conditions, 74% yield and 39,000 TTN were observed (left bars). Secondly, catalyst loading was reduced by using cells at OD$_{600}$=10, with 20 mM *N*-vinylphthalimide and 20 mM EDA. Under these conditions, 57% yield and 41,000 TTN were observed (right bars). In both cases, *dr* and *ee* were essentially unchanged. Results are the average of duplicate reactions.

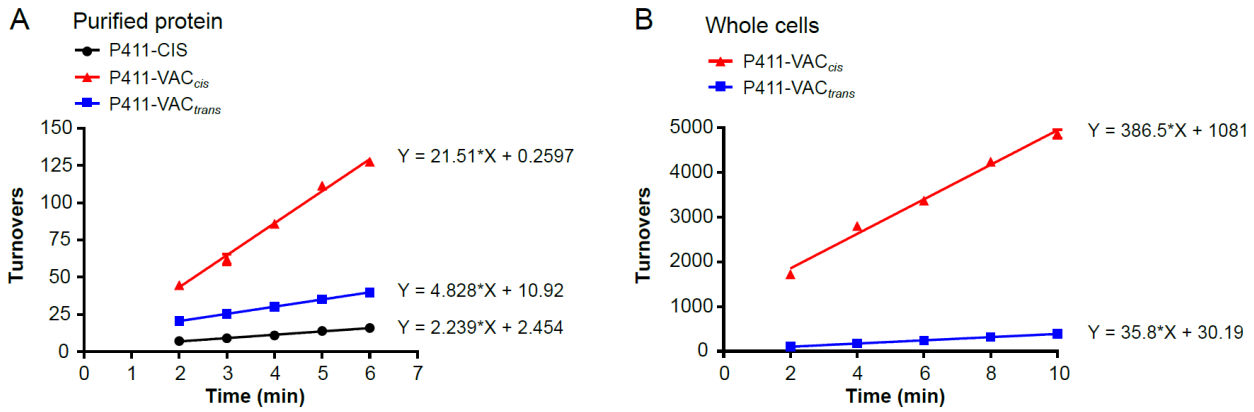


Figure S5: Determination of initial rates. (A) Apparent initial rates estimated with purified P411 variants. Reactions contained 2.5 µM P411, 5 mM NADPH, 5 mM *N*-vinylphthalimide and 5 mM EDA in M9-N buffer. (B) Apparent initial rates estimated with whole cells expressing the P411 variants. Reactions contained cells at OD_{600}=30 in M9-N buffer and 30 or 5 mM *N*-vinylphthalimide with 60 or 10 mM EDA for the *cis*- or *trans*-selective variants P411-VAC_{cis} or P411-VAC_{trans}, respectively. Reactions were performed under anaerobic conditions. Results are the average of duplicate reactions at each time point.

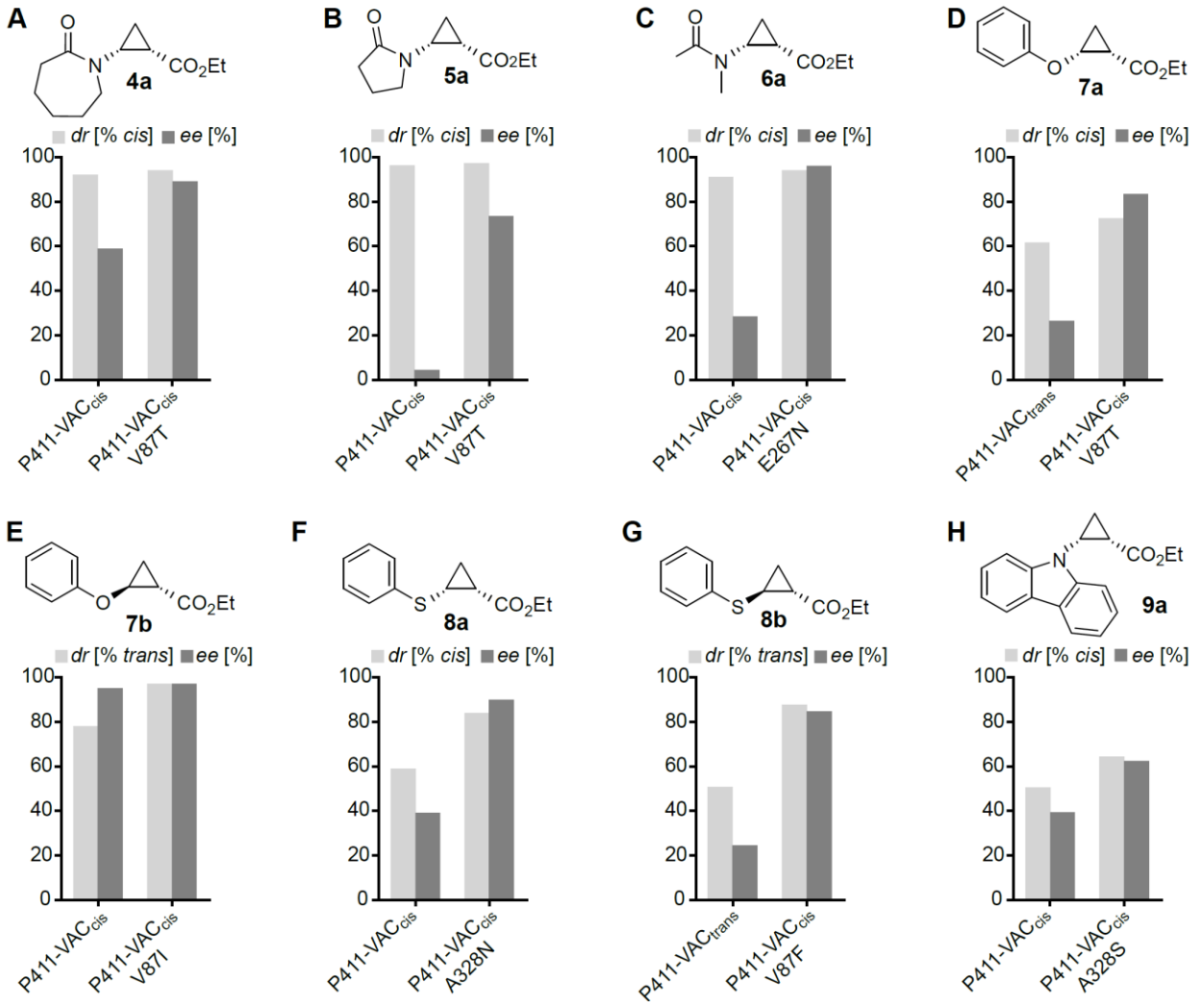


Figure S6: Improving diastereo- and enantioselectivities of P411-VAC$_{cis}$ across diverse substrates by screening a small, focused library. Shown are *dr* and *ee* obtained with P411-VAC$_{cis}$ and variants thereof containing single additional active site mutations identified in the screen of the small, focused library shown in Table S5. (A) *cis*-selective *N*-vinylcaprolactam cyclopropanation; (B) *cis*-selective *N*-vinylpyrrolidone cyclopropanation; (C) *cis*-selective *N*-methyl-*N*-vinylacetamide cyclopropanation; (D) *cis*-selective phenyl vinyl ether cyclopropanation; (E) *trans*-selective phenyl vinyl ether cyclopropanation; (F) *cis*-selective phenyl vinyl sulfide cyclopropanation; (G) *trans*-selective phenyl vinyl sulfide cyclopropanation; (H) *cis*-selective *N*-vinylcarbazole cyclopropanation. For all substrates tested, variants showing enhanced

diastereo- or enantioselectivities compared to P411-VAC$_{cis}$ could be identified. Of note, this screening approach also resulted in the identification of two P411-VAC$_{cis}$ variants with *trans* diastereoselectivity for substrates phenyl vinyl ether (E) and phenyl vinyl sulfide (G).

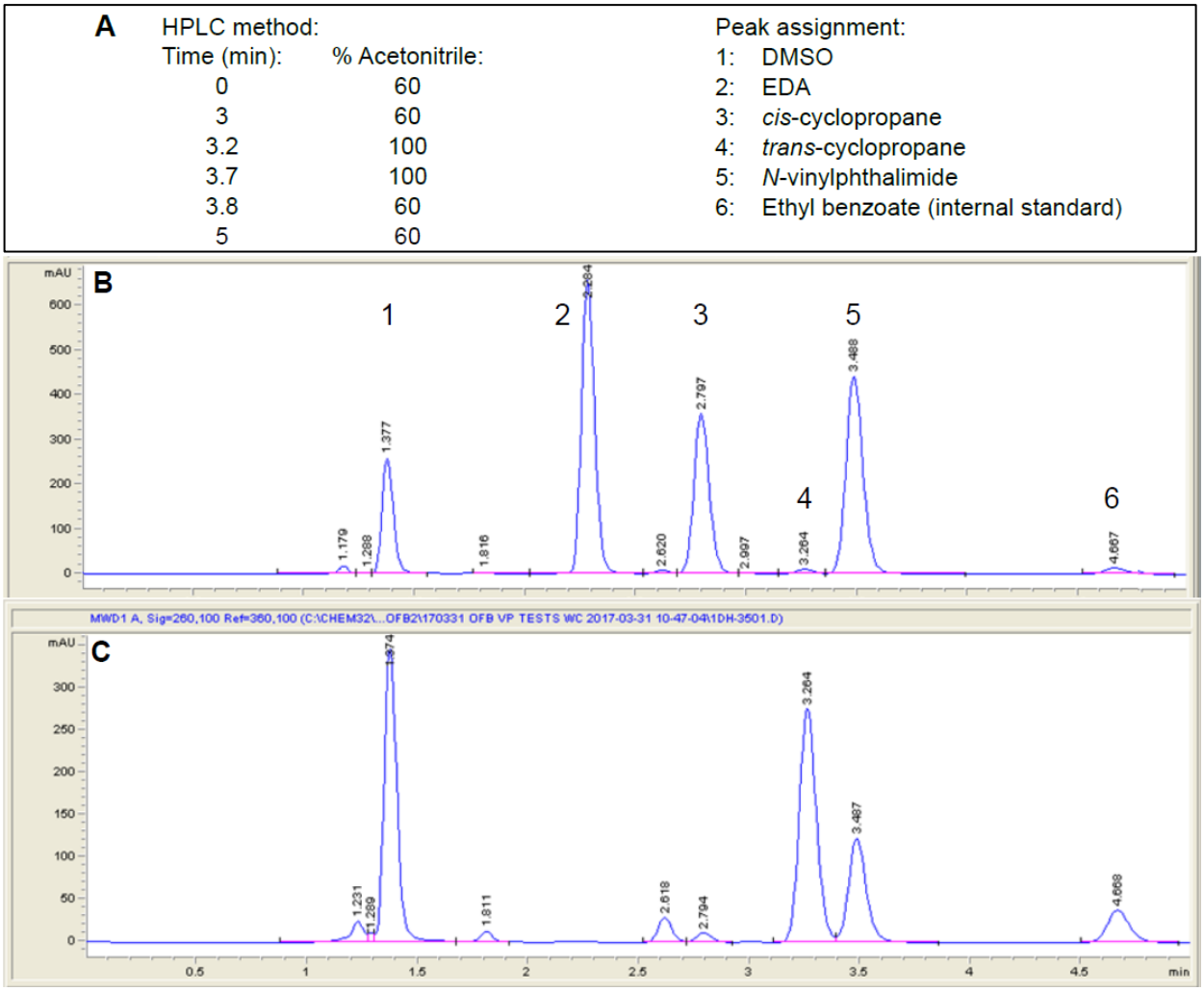


Figure S7: HPLC analysis parameters and example chromatograms. (A) Chromatography method employed on an Agilent Eclipse XDB-C18 column to analyze *N*-vinylphthalimide cyclopropanation reactions. Retention times of reaction components are indicated. (B) Exemplary HPLC run with P411 variant P411-VAC$_{cis}$. Note that this sample was taken before the reaction was complete, hence large amounts of starting materials EDA and *N*-vinylphthalimide are present. (C) Exemplary HPLC run with P411 variant P411-VAC$_{trans}$.

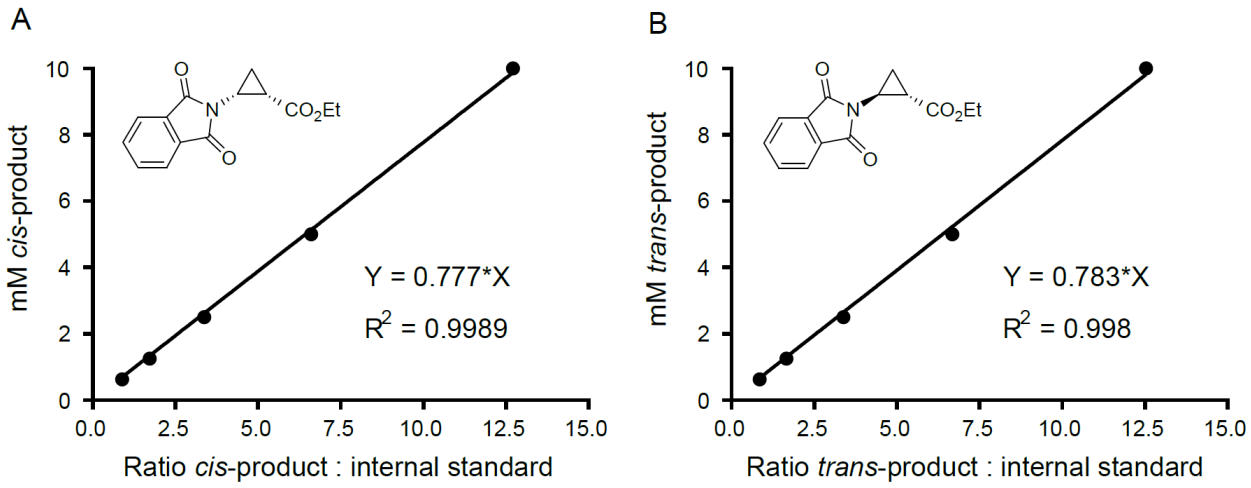
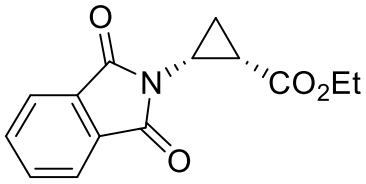


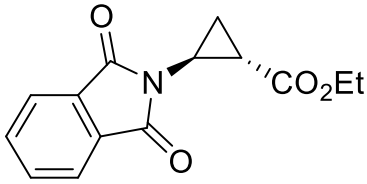
Figure S8: HPLC calibration curves. HPLC calibration curves employing ethyl benzoate as internal standard for (A) the *cis* and (B) the *trans* *N*-vinylphthalimide cyclopropanation products.

IV. Characterization of reaction products

Reactions were performed according to the general protocol described above (*Preparative scale reactions*). Spectral data of known compounds are in agreement with reported values.[14-19]



3a: Ethyl (1*S*, 2*R*)-2-(1,3-dioxoisoindolin-2-yl)cyclopropane-1-carboxylate. The reaction was performed on 4.0 mmol scale with whole-cell catalysts at OD_{600}=50 in 90 mL M9-N buffer expressing P411-VAC_{cis} at 1.2 μM, with 40 mM *N*-vinylphthalimide (added as 5 mL of a 800 mM solution in DMSO) and 60 mM EDA (added as 5 mL of a 1.2 M solution in EtOH). The product was purified by silica gel chromatography (0 to 30% EtOAc/hexanes) and isolated as a clear oil (996 mg, 96% yield, 32,000 TTN, 97:3 *dr*, 97% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.81 (dd, *J* = 5.4, 3.1 Hz, 2H), 7.69 (dd, *J* = 5.4, 3.1 Hz, 2H), 4.02 (q, *J* = 7.1 Hz, 2H), 3.01 (ddd, *J* = 7.8, 7.1, 5.8 Hz, 1H), 2.18 (dt, *J* = 8.8, 6.9 Hz, 1H), 1.94 (q, *J* = 6.3 Hz, 1H), 1.63 (ddd, *J* = 8.8, 7.7, 6.3 Hz, 1H), 1.16 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.60, 168.69, 134.19, 131.75, 123.43, 61.08, 27.62, 19.04, 14.16, 13.07. HRMS (FAB+) exact mass calculated for $C_{14}H_{14}NO_4^+$ requires *m/z* 260.0923, found 260.0915.

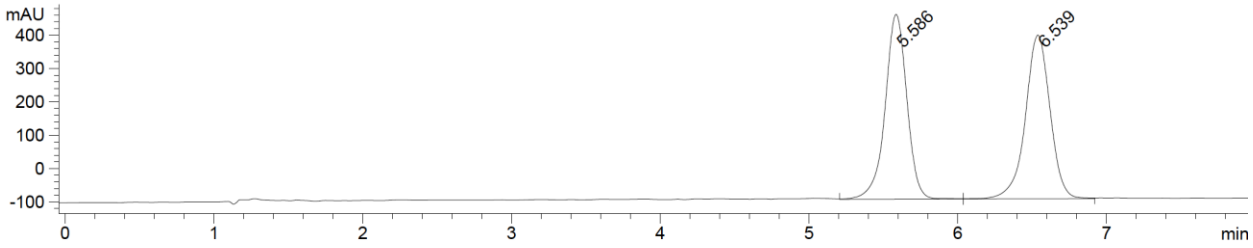


3b: Ethyl (1*S*, 2*S*)-2-(1,3-dioxoisoindolin-2-yl)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC_{trans} at 7.2 μM, with 5 mM *N*-vinylphthalimide (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 30% EtOAc/hexanes) and isolated as a white solid (28.5 mg, 88% yield, 610 TTN, 3:97 *dr*, 92% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.82 (dd, *J* = 5.5, 3.0 Hz, 2H), 7.72 (dd, *J* = 5.5, 3.0 Hz, 2H), 4.20 (q, *J* = 7.2 Hz, 2H), 3.30 (ddd, *J* = 8.2, 5.2, 3.1

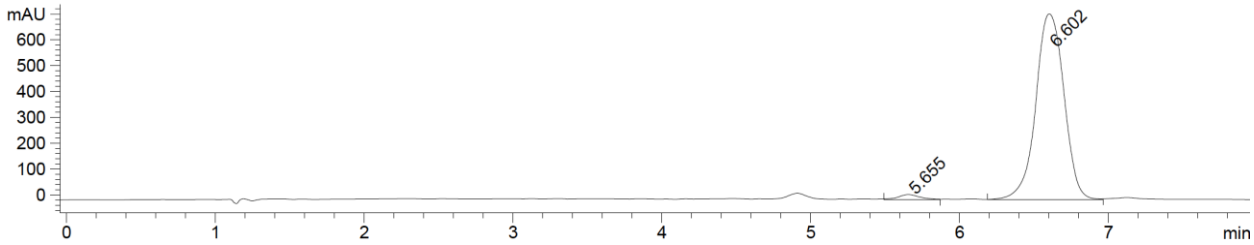
Hz, 1H), 2.21 (ddd, *J* = 9.3, 6.2, 3.2 Hz, 1H), 1.75 (ddd, *J* = 9.3, 5.7, 5.2 Hz, 1H), 1.63 (ddd, *J* = 8.1, 6.2, 5.8 Hz, 1H), 1.30 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.26, 168.16, 134.37, 131.65, 123.48, 61.23, 29.61, 20.09, 14.35, 13.69. HRMS (FAB+) exact mass calculated for $C_{14}H_{14}NO_4^+$ requires *m/z* 260.0923, found 260.0928.

Determination of *N*-vinylphthalimide cyclopropanation enantioselectivity. Reaction mixtures were extracted with cyclohexane, or purified compounds were dissolved in 20% isopropanol in hexanes, and samples were analyzed by chiral SFC using a Chiralpak AD-H column (10% isopropanol, isocratic).

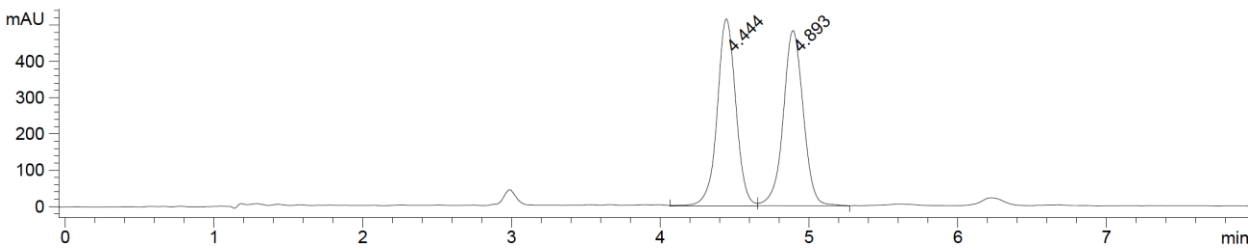
Racemic *cis* isomer, chemically prepared:



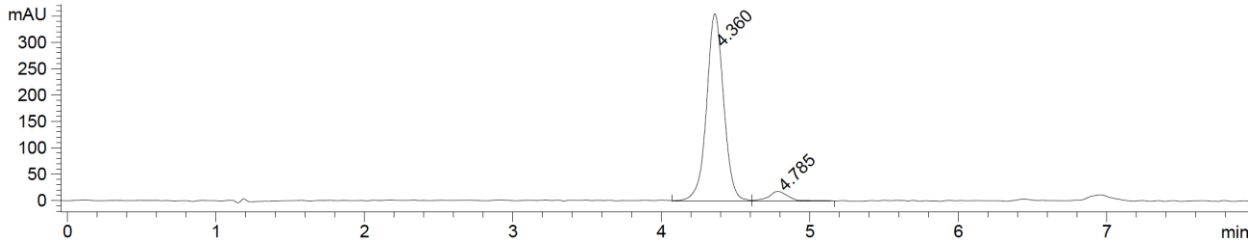
Enzymatic reaction with P411-VAC$_{cis}$ (97% *ee cis* (1*S*, 2*R*) isomer):

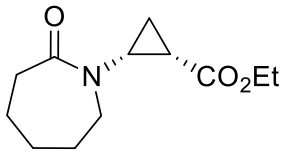


Racemic *trans* isomer, chemically prepared:

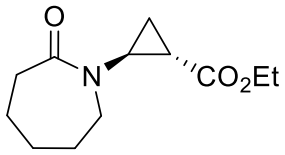


Enzymatic reaction, with P411-VAC$_{trans}$ (92% *ee trans* (1*S*, 2*S*) isomer):





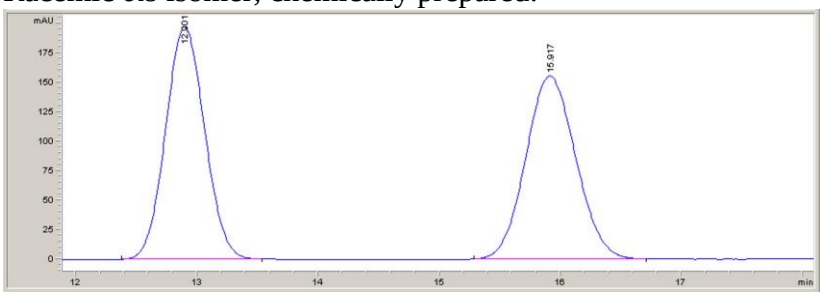
4a: Ethyl (1*S*, 2*R*)-2-(2-oxoazepan-1-yl)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC$_{cis}$ V87T at 1.5 μM, with 5 mM *N*-vinylcaprolactam (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 100% EtOAc/hexanes) and isolated as a clear oil (21.8 mg, 78% yield, 2600 TTN, 95:5 dr, 88% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.09 (q, *J* = 7.1 Hz, 2H), 3.48 (ddd, *J* = 15.2, 9.2, 1.6 Hz, 1H), 3.39 (ddd, *J* = 15.2, 7.6, 1.9 Hz, 1H), 3.07 (td, *J* = 7.5, 6.1 Hz, 1H), 2.45 (dd, *J* = 6.6, 4.2 Hz, 2H), 2.13 (ddd, *J* = 8.4, 7.4, 6.3 Hz, 1H), 1.91 – 1.77 (m, 2H), 1.69 – 1.56 (m, 4H), 1.37 (q, *J* = 6.2 Hz, 1H), 1.28 – 1.18 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.58, 170.59, 60.60, 51.13, 38.75, 37.58, 30.08, 27.84, 23.01, 22.98, 14.14, 13.44. HRMS (ESI) exact mass calculated for $C_{12}H_{19}NO_3^+$ requires m/z 226.1443, found 226.1413.



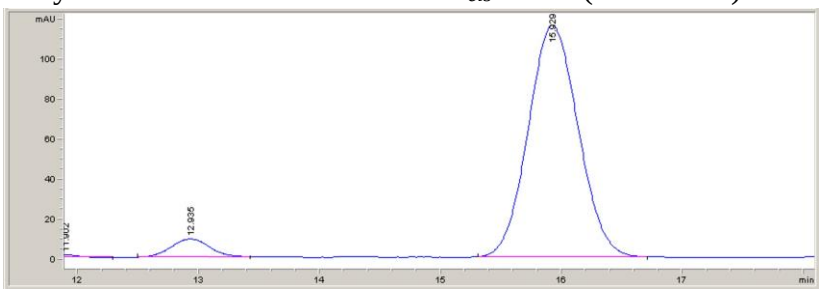
4b: Ethyl (1*S*, 2*S*)-2-(2-oxoazepan-1-yl)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC$_{trans}$ at 6.1 μM, with 5 mM *N*-vinylcaprolactam (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 100% EtOAc/hexanes) and isolated as a clear oil (17.5 mg, 62% yield, 510 TTN, 7:93 *dr*, 86% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.14 (qd, *J* = 7.1, 0.8 Hz, 2H), 3.36 (dd, *J* = 6.2, 3.9 Hz, 2H), 3.15 (ddd, *J* = 7.7, 5.3, 3.1 Hz, 1H), 2.52 – 2.45 (m, 2H), 1.78 (ddd, *J* = 9.1, 5.9, 3.1 Hz, 1H), 1.72 – 1.58 (m, 6H), 1.48 (dt, *J* = 7.7, 5.7 Hz, 1H), 1.28 – 1.19 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.51, 172.57, 60.94, 60.81, 50.31, 39.38, 37.76, 29.96, 29.16, 23.48, 23.04, 16.76, 14.35. HRMS (ESI) exact mass calculated for $C_{12}H_{19}NO_3^+$ requires *m/z* 226.1443, found 226.1425.

Determination of *N*-vinylcaprolactam cyclopropanation enantioselectivity. Reaction mixtures were extracted with cyclohexane, or purified compounds were dissolved in 20% isopropanol in hexanes, and samples were analyzed by chiral HPLC using a Chiralpak IC column (*cis* product: 40% isopropanol, isocratic, 1 mL/min flow rate; *trans* product: 50% isopropanol, isocratic, 1.2 mL/min flow rate).

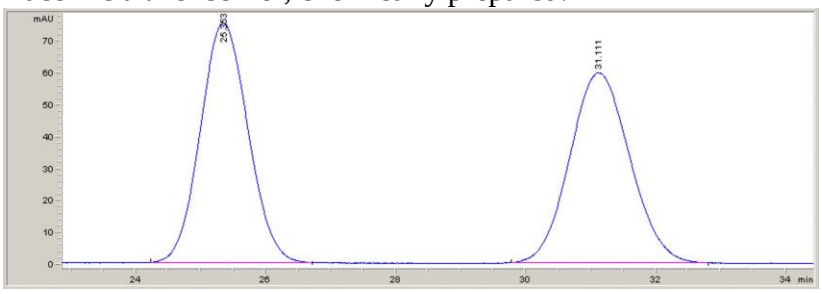
Racemic *cis* isomer, chemically prepared:



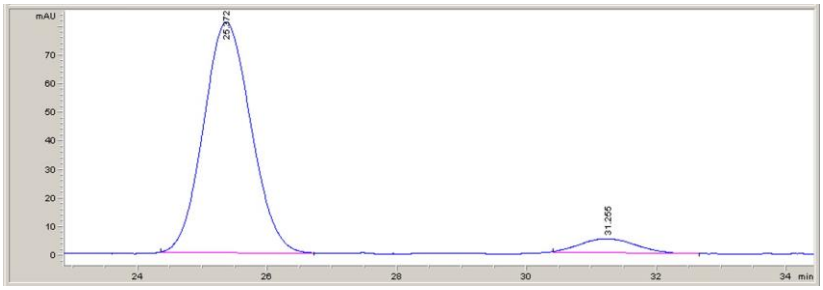
Enzymatic reaction with P411-VAC$_{cis}$ V87T (88% *ee cis*):

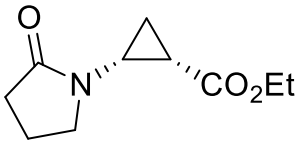


Racemic *trans* isomer, chemically prepared:

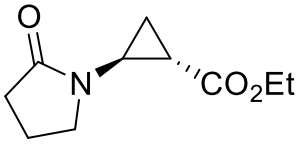


Enzymatic reaction, with P411-VAC$_{trans}$ (86% *ee trans*):





5a: Ethyl (1*S*, 2*R*)-2-(2-oxopyrrolidin-1-yl)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC_{cis} V87T at 1.5 μM, with 5 mM *N*-vinylpyrrolidone (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 100% EtOAc/hexanes) and isolated as a clear oil (14.5 mg, 59% yield, 1960 TTN, 97:3 *dr*, 74% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.10 (q, *J* = 7.2 Hz, 2H), 3.61 – 3.50 (m, 1H), 3.33 (ddd, *J* = 9.1, 7.5, 5.8 Hz, 1H), 3.00 (qt, *J* = 7.2, 0.9 Hz, 1H), 2.38 – 2.32 (m, 2H), 2.08 – 1.98 (m, 3H), 1.51 (q, *J* = 6.1 Hz, 1H), 1.31 – 1.23 (m, 4H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.08, 170.96, 60.96, 48.54, 32.82, 31.60, 20.78, 18.69, 14.29, 12.57. HRMS (ESI) exact mass calculated for $C_{10}H_{15}NO_3^+$ requires *m/z* 198.1130, found 198.1113.

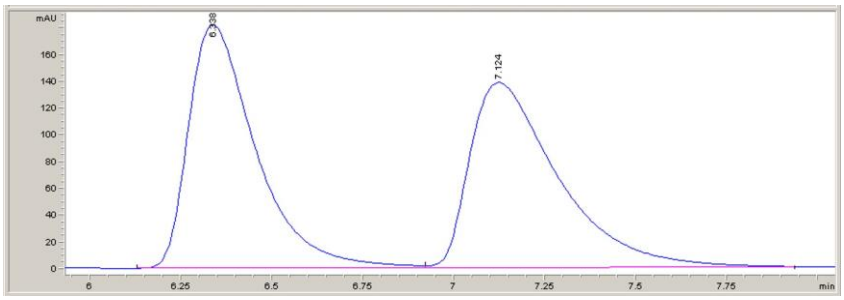


5b: Ethyl (1*S*, 2*S*)-2-(2-oxopyrrolidin-1-yl)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC_{trans} at 6.1 μM, with 5 mM *N*-vinylpyrrolidone (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 100% EtOAc/hexanes) and isolated as a clear oil (14 mg, 57% yield, 470 TTN, 5:95 *dr*, 92% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.15 (qd, *J* = 7.2, 1.5 Hz, 2H), 3.36 – 3.29 (m, 2H), 3.18 (dddt, *J* = 8.0, 5.3, 3.1, 0.8 Hz, 1H), 2.40 (t, *J* = 8.1 Hz, 2H), 2.05 – 1.97 (m, 2H), 1.86 (ddd, *J* = 9.1, 5.9, 3.1 Hz, 1H), 1.48 (dt, *J* = 8.0, 5.8 Hz, 1H), 1.42 (dt, *J* = 9.2, 5.5 Hz, 1H), 1.28 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 176.14, 172.38, 61.03, 47.38, 34.15, 31.77, 19.86, 18.12, 14.29, 14.20. HRMS (ESI) exact mass calculated for $C_{10}H_{15}NO_3^+$ requires *m/z* 198.1130, found 198.1140.

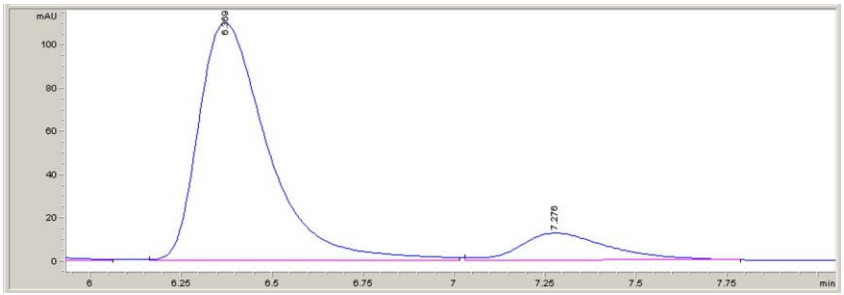
Determination of *N*-vinylpyrrolidone cyclopropanation enantioselectivity. Reaction mixtures were extracted with cyclohexane, or purified compounds were dissolved in 20% isopropanol in

hexanes, and samples were analyzed by chiral HPLC. *Cis* product: Chiralpak IA column, 20% isopropanol, isocratic, 1 mL/min flow rate; *trans* product: Chiralpak OJ-H column, 5% isopropanol, isocratic, 1 mL/min flow rate.

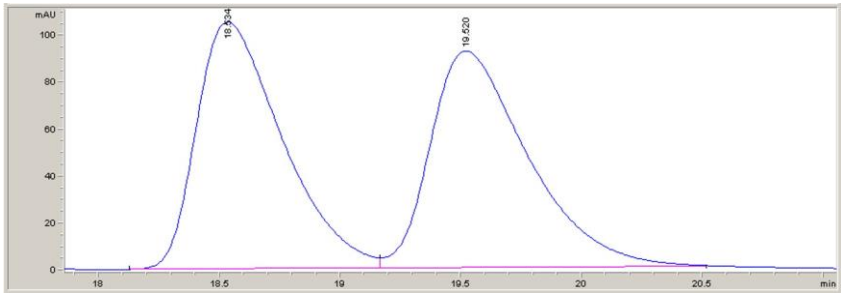
Racemic *cis* isomer, chemically prepared:



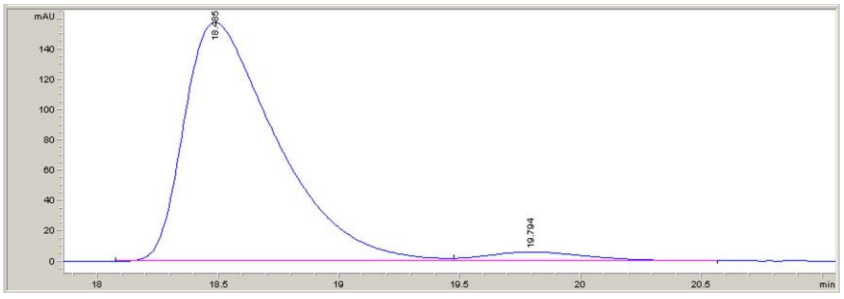
Enzymatic reaction with P411-VAC$_{cis}$ V87T (74% *ee cis*):

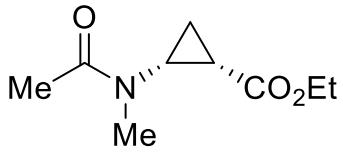


Racemic *trans* isomer, chemically prepared:

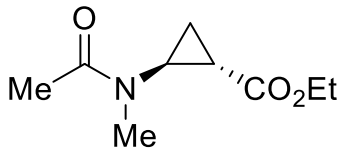


Enzymatic reaction, with P411-VAC$_{trans}$ (92% *ee trans*):





6a: Ethyl (1*S*, 2*R*)-2-(*N*-methylacetamido)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC$_{cis}$ E267N at 1.1 μM, with 5 mM *N*-methyl-*N*-vinylacetamide (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 100% EtOAc/hexanes) and isolated as a clear oil (8.5 mg, 37% yield, 1700 TTN, 94:6 *dr*, 97% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) Mixture of rotamers: δ 4.19 – 4.03 (m, 2H), 3.20 – 3.06 (m, 1H), 3.04 – 2.88 (m, 3H), 2.19 – 1.98 (m, 4H), 1.74 – 1.67 (m, 1H), 1.46 – 1.34 (m, 1H), 1.29 – 1.19 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.76, 172.62, 171.02, 169.61, 61.03, 60.59, 39.40, 37.93, 36.88, 33.92, 23.33, 22.26, 22.09, 21.85, 14.49, 14.12, 14.05, 13.52. HRMS (ESI) exact mass calculated for $C_9H_{15}NO_3^+$ requires *m/z* 186.1130, found 186.1120.

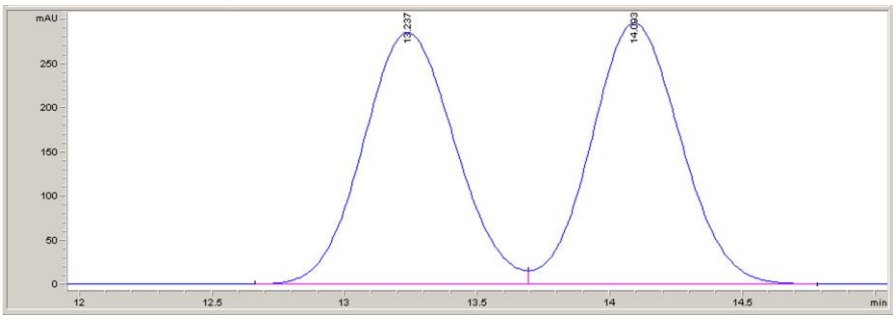


6b: Ethyl (1*S*, 2*S*)-2-(*N*-methylacetamido)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC$_{trans}$ at 6.9 μM, with 5 mM *N*-methyl-*N*-vinylacetamide (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 100% EtOAc/hexanes) and isolated as a clear oil (15 mg, 65% yield, 470 TTN, 4:96 *dr*, 95% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 4.17 (q, *J* = 7.1 Hz, 2H), 3.15 (ddd, *J* = 7.8, 5.1, 3.0 Hz, 1H), 2.89 (s, 3H), 2.16 (s, 3H), 1.91 (ddd, *J* = 9.2, 5.9, 3.0 Hz, 1H), 1.54 (dt, *J* = 7.7, 5.7 Hz, 1H), 1.33 (dt, *J* = 9.4, 5.3 Hz, 1H), 1.27 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 173.18, 171.73, 61.29, 40.20, 33.77, 24.22, 22.56, 17.66, 14.36. HRMS (ESI) exact mass calculated for $C_9H_{15}NO_3^+$ requires *m/z* 186.1130, found 186.1100.

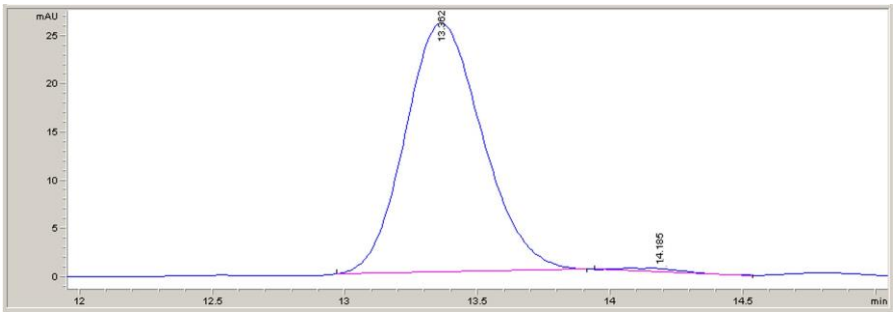
Determination of *N*-methyl-*N*-vinylacetamide cyclopropanation enantioselectivity. Reaction mixtures were extracted with cyclohexane, or purified compounds were dissolved in 20%

isopropanol in hexanes, and samples were analyzed by chiral HPLC using a Chiralpak IC column, 40% isopropanol, isocratic, 1 mL/min flow rate.

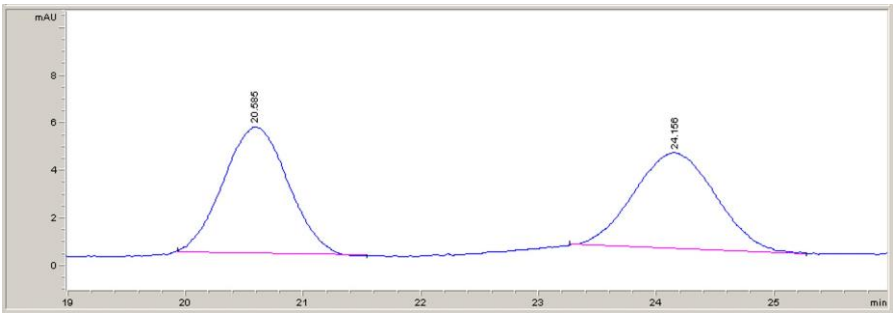
Racemic *cis* isomer, chemically prepared:



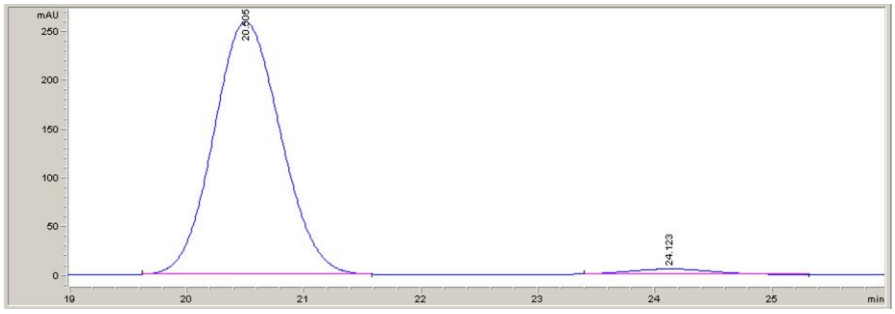
Enzymatic reaction with P411-VAC$_{cis}$ E267N (97% *ee cis*):

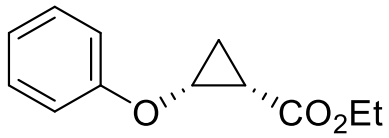


Racemic *trans* isomer, chemically prepared:

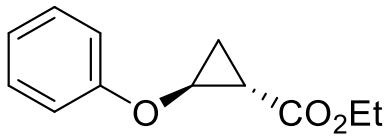


Enzymatic reaction, with P411-VAC$_{trans}$ (95% *ee trans*):





7a: Ethyl *cis*-2-phenoxycyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC_{cis} V87T at 1.5 μM, with 5 mM phenyl vinyl ether (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 30% EtOAc/hexanes) and isolated as a clear oil (17 mg, 66% yield, 2200 TTN, 72:28 *dr*, 87% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.30 – 7.24 (m, 2H), 7.03 (dd, *J* = 8.8, 1.1 Hz, 2H), 6.99 – 6.94 (m, 1H), 4.03 – 3.95 (m, 3H), 2.02 (dt, *J* = 8.8, 6.8 Hz, 1H), 1.77 (ddd, *J* = 6.9, 6.3, 4.6 Hz, 1H), 1.32 (dt, *J* = 8.8, 6.5 Hz, 1H), 1.04 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.21, 158.24, 129.49, 121.66, 115.09, 60.83, 55.61, 21.49, 14.15, 12.98. HRMS (EI+) exact mass calculated for $C_{12}H_{14}O_3^+$ requires *m/z* 206.0943, found 206.0925.

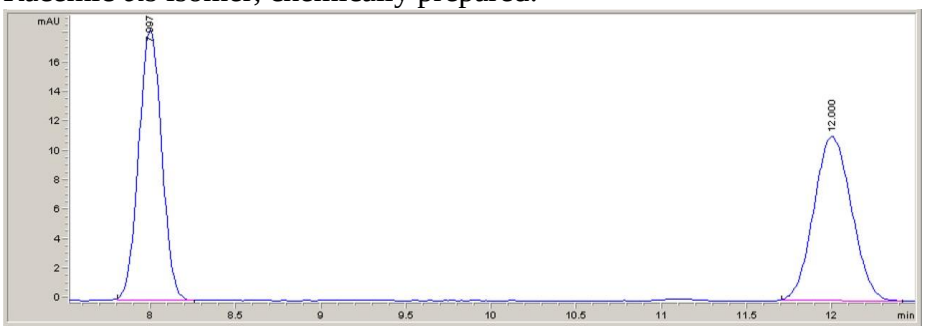


7b: Ethyl *trans*-2-phenoxycyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC_{cis} V87I at 1.3 μM, with 5 mM phenyl vinyl ether (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 30% EtOAc/hexanes) and isolated as a clear oil (19 mg, 74% yield, 2800 TTN, 2:98 *dr*, 94% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.27 (m, 2H), 7.03 – 6.95 (m, 3H), 4.20 (qd, *J* = 7.1, 2.4 Hz, 2H), 4.07 (ddd, *J* = 6.5, 4.1, 2.1 Hz, 1H), 1.95 (ddd, *J* = 9.8, 6.2, 2.1 Hz, 1H), 1.51 (dt, *J* = 6.7, 6.0 Hz, 1H), 1.42 (ddd, *J* = 9.9, 5.8, 4.1 Hz, 1H), 1.30 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.25, 158.06, 129.67, 121.79, 114.99, 61.02, 57.28, 21.77, 15.78, 14.43. HRMS (EI+) exact mass calculated for $C_{12}H_{14}O_3^+$ requires *m/z* 206.0943, found 206.0917.

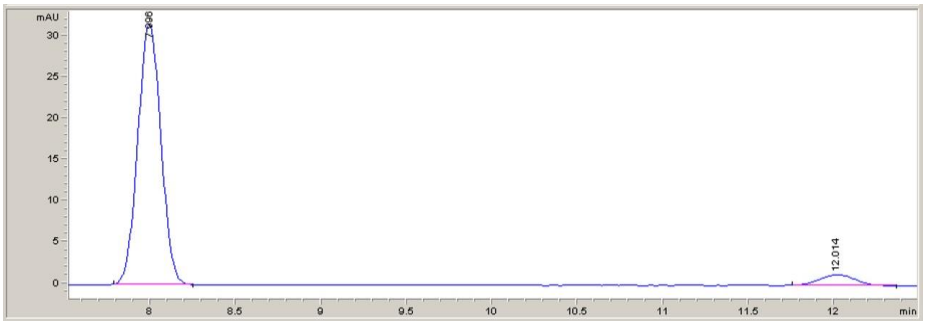
Determination of phenyl vinyl ether cyclopropanation enantioselectivity. Reaction mixtures were extracted with cyclohexane, or purified compounds were dissolved in 20% isopropanol in

hexanes, and samples were analyzed by chiral HPLC using a Chiralpak IC column (*trans* product: 1% isopropanol, isocratic, 1 mL/min flow rate; *cis* product: 7.5% isopropanol, isocratic, 1 mL/min flow rate).

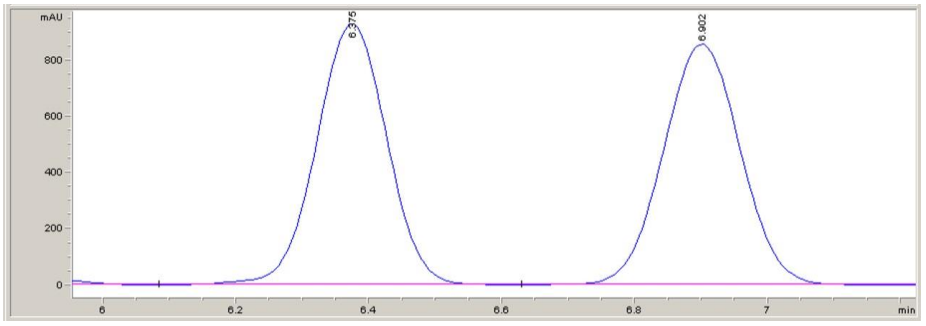
Racemic *cis* isomer, chemically prepared:



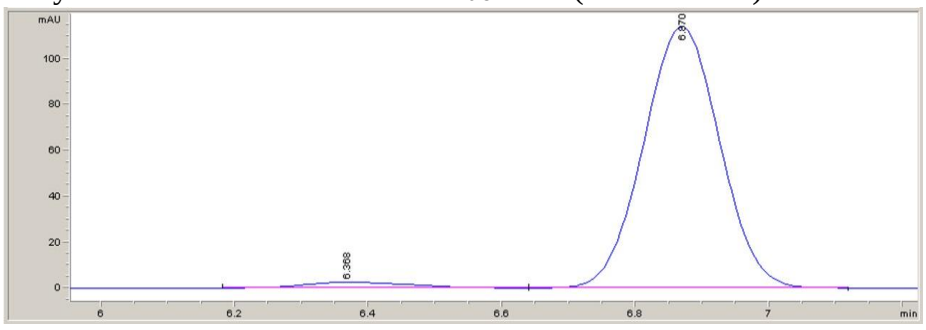
Enzymatic reaction, with P411-VAC$_{cis}$ V87T (87% *ee cis*):

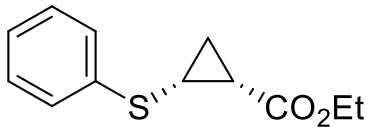


Racemic *trans* isomer, chemically prepared:

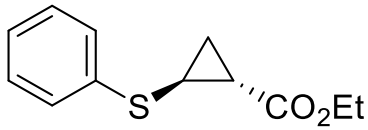


Enzymatic reaction with P411-VAC$_{cis}$ V87I (94% *ee trans*):





8a: Ethyl *cis*-2-(phenylthio)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC_{cis} A328N at 1.6 μM, with 5 mM phenyl vinyl sulfide (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 30% EtOAc/hexanes) and isolated as a clear oil (12 mg, 43% yield, 1400 TTN, 84:16 *dr*, 90% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.38 – 7.32 (m, 2H), 7.30 – 7.22 (m, 2H), 7.17 – 7.10 (m, 1H), 4.04 (q, *J* = 7.1 Hz, 2H), 2.69 (td, *J* = 7.9, 6.9 Hz, 1H), 2.23 (td, *J* = 7.7, 6.7 Hz, 1H), 1.49 – 1.43 (m, 2H), 1.09 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.81, 137.27, 128.87, 127.60, 125.65, 60.98, 22.22, 22.18, 14.24, 13.33. HRMS (EI+) exact mass calculated for $C_{12}H_{14}O_2S$ requires *m/z* 222.0715, found 222.0717.

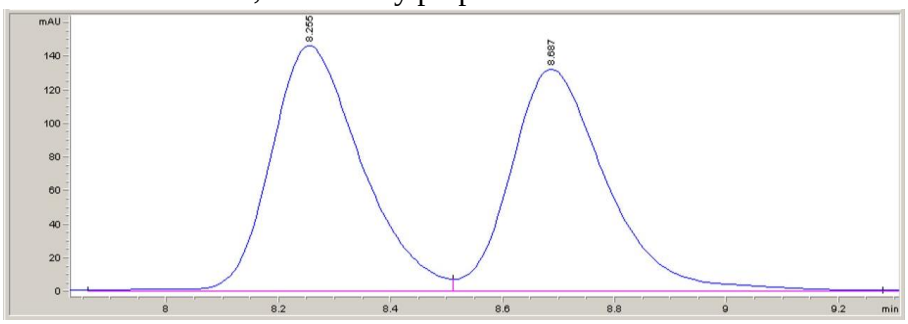


8b: Ethyl *trans*-2-(phenylthio)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC_{cis} V87F at 1.7 μM, with 5 mM phenyl vinyl sulfide (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 30% EtOAc/hexanes) and isolated as a clear oil (16 mg, 58% yield, 1700 TTN, 13:87 *dr*, 84% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.34 – 7.27 (m, 4H), 7.21 – 7.15 (m, 1H), 4.18 (qd, *J* = 7.1, 6.1 Hz, 2H), 2.76 (ddd, *J* = 8.2, 5.6, 3.6 Hz, 1H), 1.91 (ddd, *J* = 8.8, 5.4, 3.6 Hz, 1H), 1.66 (ddd, *J* = 8.2, 5.4, 4.8 Hz, 1H), 1.28 (t, *J* = 7.1 Hz, 3H), 1.25 – 1.20 (m, 1H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.47, 136.96, 129.08, 127.35, 125.87, 61.14, 24.33, 22.44, 17.42, 14.40. HRMS (EI+) exact mass calculated for $C_{12}H_{14}O_2S$ requires *m/z* 222.0715, found 222.0706.

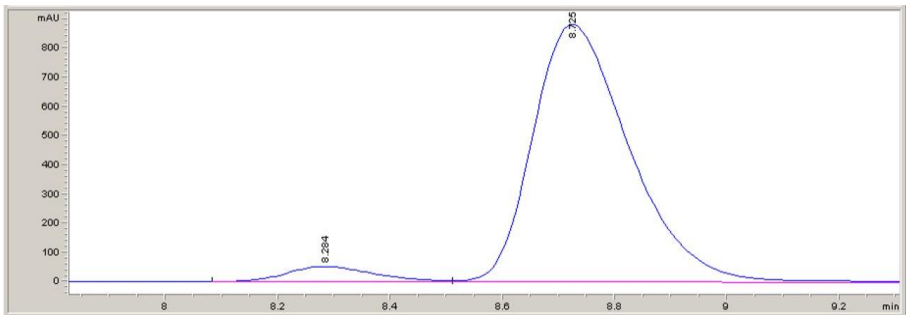
Determination of phenyl vinyl sulfide cyclopropanation enantioselectivity. Reaction mixtures were extracted with cyclohexane, or purified compounds were dissolved in 20% isopropanol in

hexanes, and samples were analyzed by chiral HPLC using a Chiralpak IA column, 1% isopropanol, isocratic, 1 mL/min flow rate.

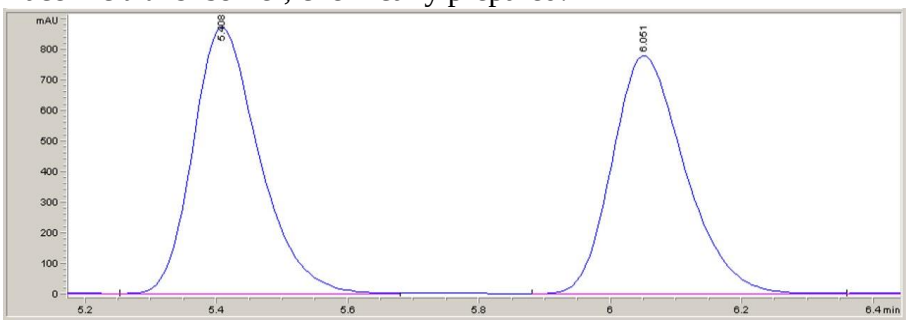
Racemic *cis* isomer, chemically prepared:



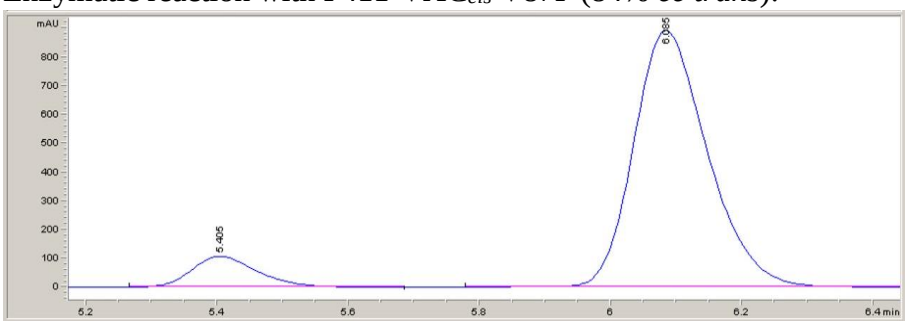
Enzymatic reaction, with P411-VAC$_{cis}$ A328N (90% *ee cis*):

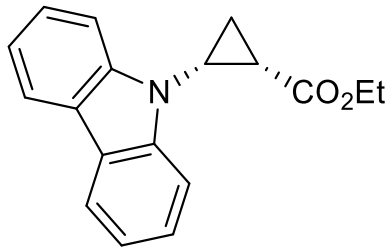


Racemic *trans* isomer, chemically prepared:

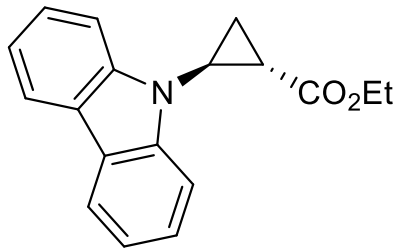


Enzymatic reaction with P411-VAC$_{cis}$ V87F (84% *ee trans*):





9a: Ethyl *cis*-2-(9H-carbazol-9-yl)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC_{cis} A328S at 1.4 μM, with 5 mM N-vinylcarbazole (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 30% EtOAc/hexanes) and isolated as a white solid (13 mg, 37% yield, 1300 TTN, 64:36 *dr*, 62% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (d, *J* = 7.7 Hz, 2H), 7.66 – 7.48 (m, 2H), 7.43 (t, *J* = 7.6 Hz, 2H), 7.22 (ddd, *J* = 8.0, 7.2, 1.0 Hz, 2H), 3.75 (td, *J* = 7.2, 5.7 Hz, 1H), 3.61 (qd, *J* = 7.1, 2.1 Hz, 2H), 2.40 (ddd, *J* = 8.6, 7.0, 6.4 Hz, 1H), 2.20 (dt, *J* = 6.4, 5.7 Hz, 1H), 1.84 (ddd, *J* = 8.6, 7.4, 5.5 Hz, 1H), 0.69 (t, *J* = 7.1 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.69, 141.41, 125.75, 123.26, 120.40, 119.57, 109.88, 60.85, 31.97, 21.47, 14.06, 13.57. HRMS (FAB+) exact mass calculated for $C_{18}H_{17}NO_2^+$ requires *m/z* 280.1338, found 280.1310.

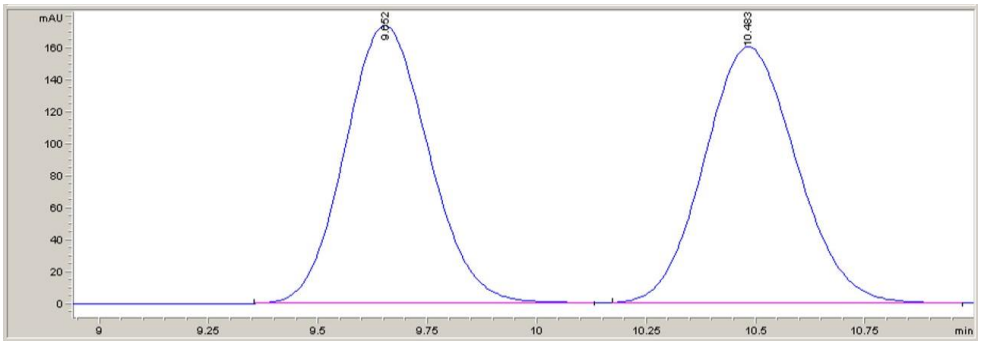


9b: Ethyl *trans*-2-(9H-carbazol-9-yl)cyclopropane-1-carboxylate. The reaction was performed on 0.125 mmol scale with whole-cell catalysts at OD_{600}=50 in 23 mL M9-N buffer expressing P411-VAC_{trans} at 7.6 μM, with 5 mM *N*-vinylcarbazole (added as 1 mL of a 125 mM solution in DMSO) and 10 mM EDA (added as 1 mL of a 250 mM solution in EtOH). The product was purified by silica gel chromatography (0 to 30% EtOAc/hexanes) and isolated as a clear oil (19 mg, 54% yield, 360 TTN, 6:94 *dr*, 94% *ee*). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.04 (ddd, *J* = 7.8, 1.1, 0.7 Hz, 2H), 7.54 (dt, *J* = 8.2, 0.8 Hz, 2H), 7.45 (ddd, *J* = 8.2, 7.1, 1.2 Hz, 2H), 7.24 (ddd, *J* = 8.0, 7.1, 1.0 Hz, 2H), 4.32 (qd, *J* = 7.1, 2.0 Hz, 2H), 3.72 (ddd, *J* = 7.5, 4.8, 2.8 Hz, 1H), 2.27 (ddd, *J* = 9.3,

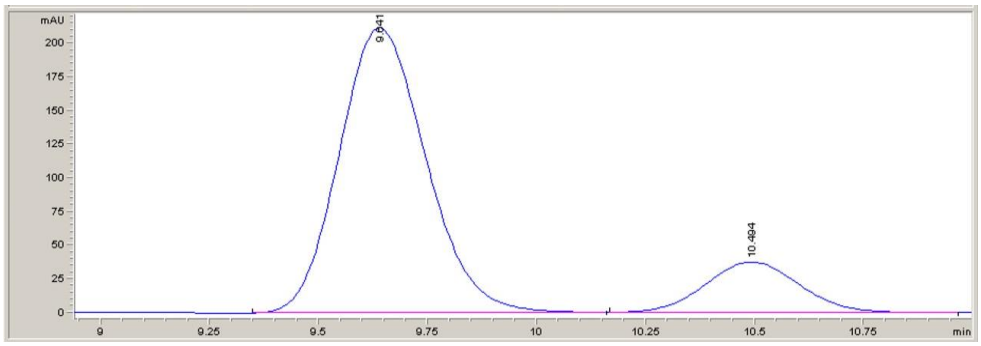
5.9, 2.8 Hz, 1H), 1.95 (ddd, *J* = 7.3, 5.9, 5.1 Hz, 1H), 1.69 (dt, *J* = 9.2, 5.0 Hz, 1H), 1.38 (t, *J* = 7.2 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 172.07, 140.92, 126.05, 123.28, 120.47, 119.83, 109.89, 61.44, 32.73, 22.27, 16.17, 14.53. HRMS (FAB+) exact mass calculated for $C_{18}H_{17}NO_2^+$ requires *m/z* 280.1338, found 280.1322.

Determination of *N*-vinylcarbazole cyclopropanation enantioselectivity. Reaction mixtures were extracted with cyclohexane, or purified compounds were dissolved in 20% isopropanol in hexanes, and samples were analyzed by chiral HPLC using a Chiralpak IC column (*cis* product: 5% isopropanol, isocratic, 1 mL/min flow rate; *trans* product: 1% isopropanol, isocratic, 1 mL/min flow rate).

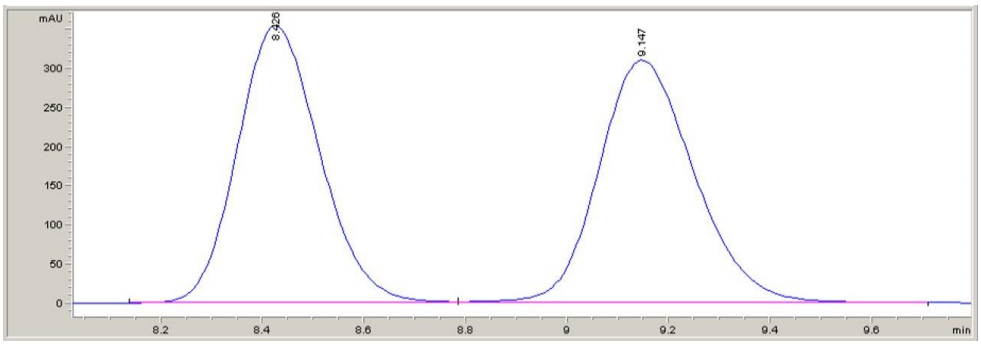
Racemic *cis* isomer, chemically prepared:



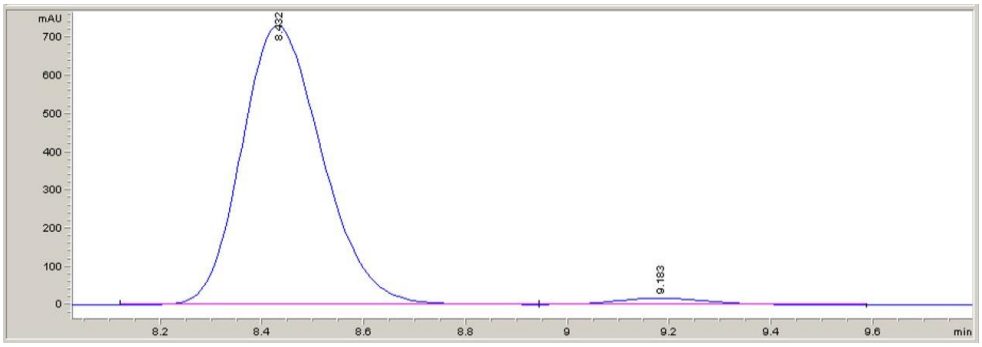
Enzymatic reaction with P411-VAC$_{cis}$ A328S (62% *ee cis*):



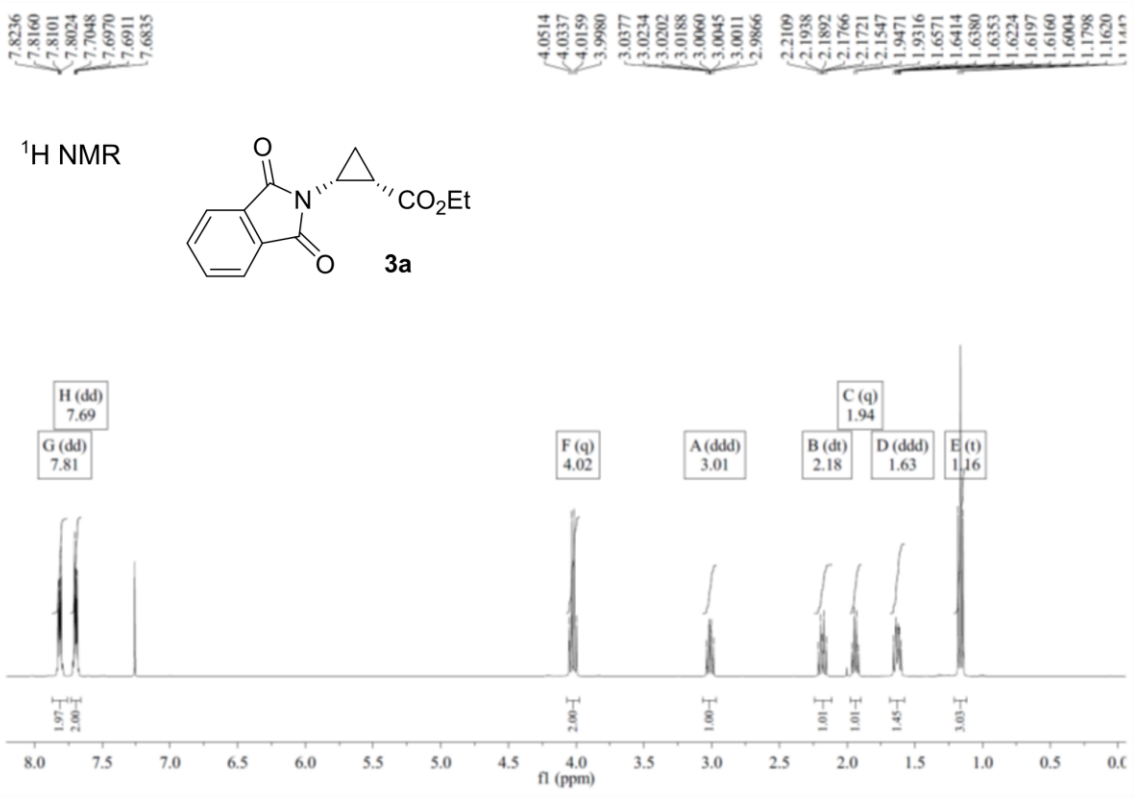
Racemic *trans* isomer, chemically prepared:

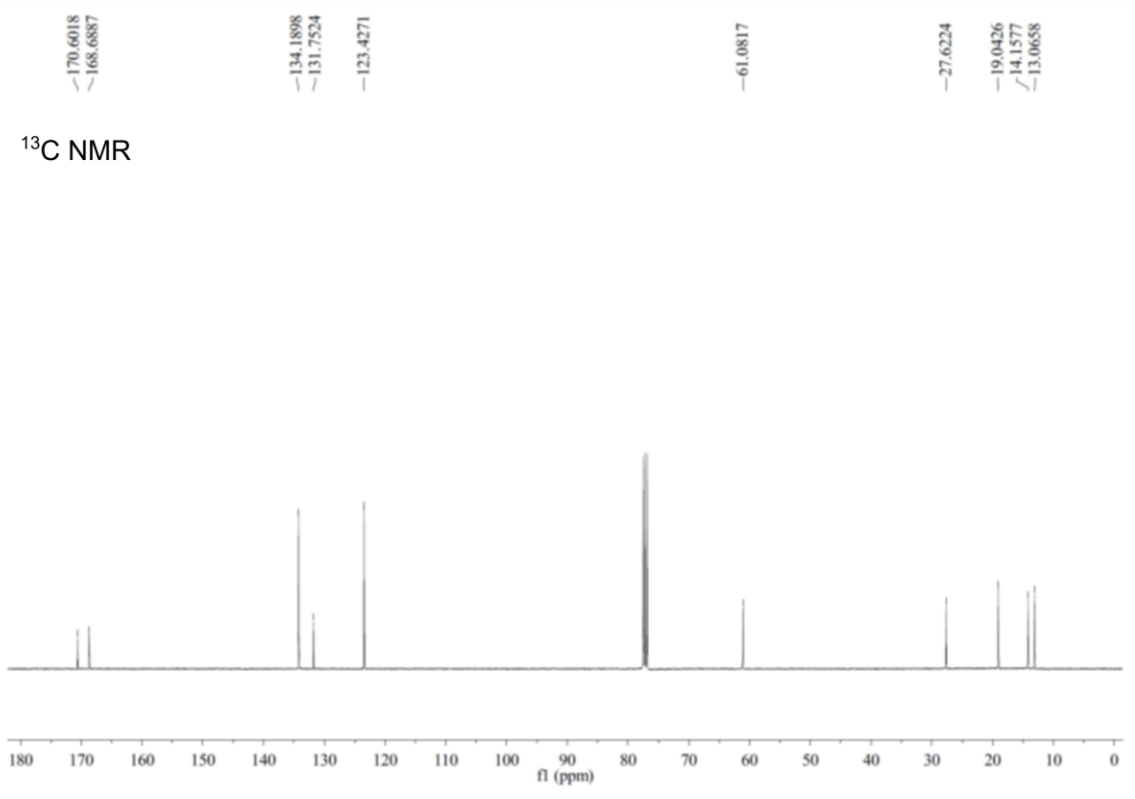


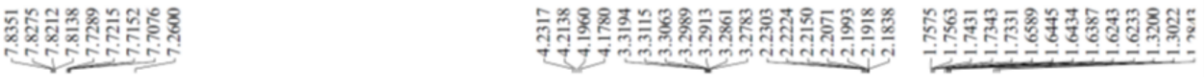
Enzymatic reaction, with P411-VAC$_{trans}$ (94% *ee trans*):



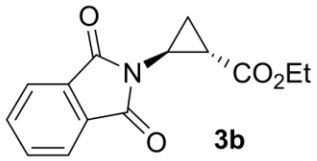
V. NMR spectra



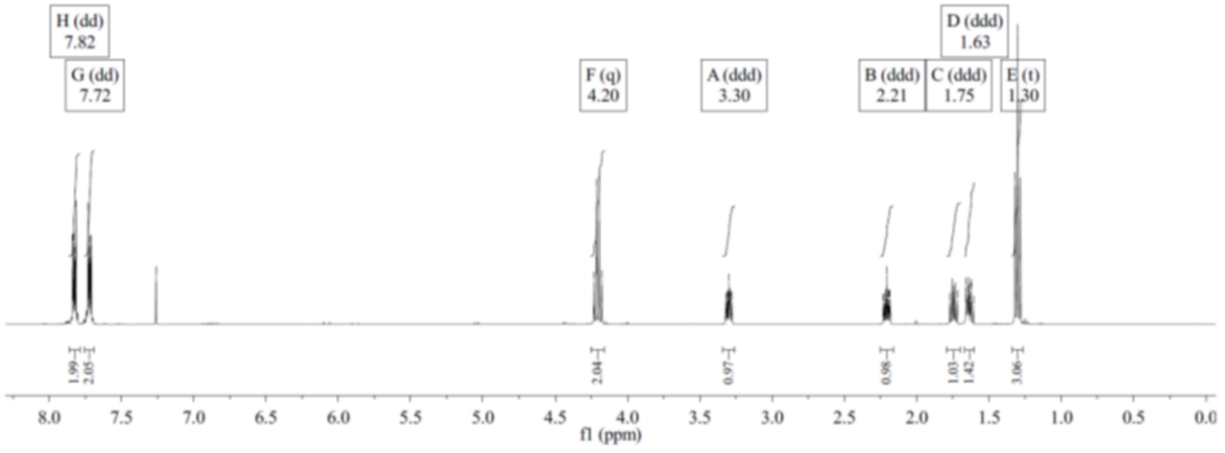




^{1}H NMR



3b



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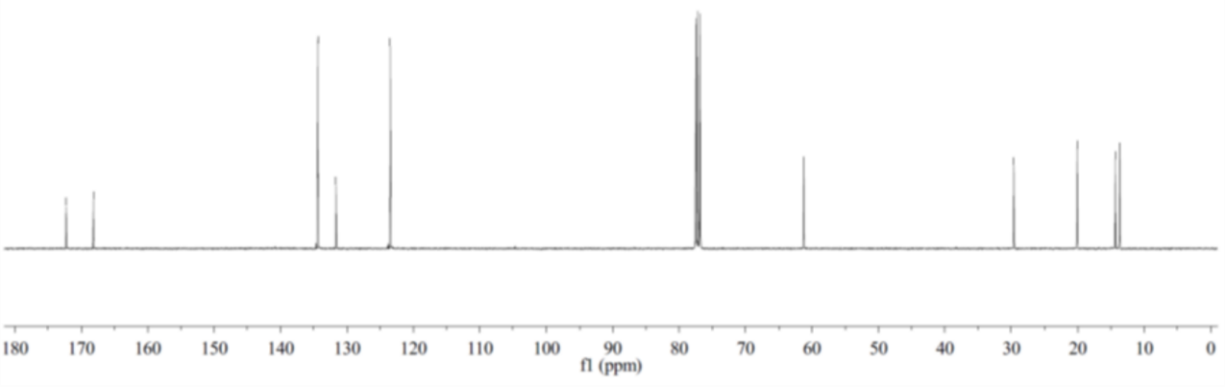

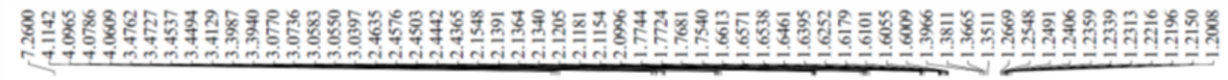

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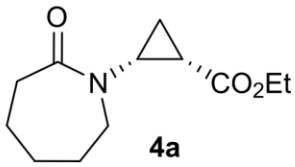

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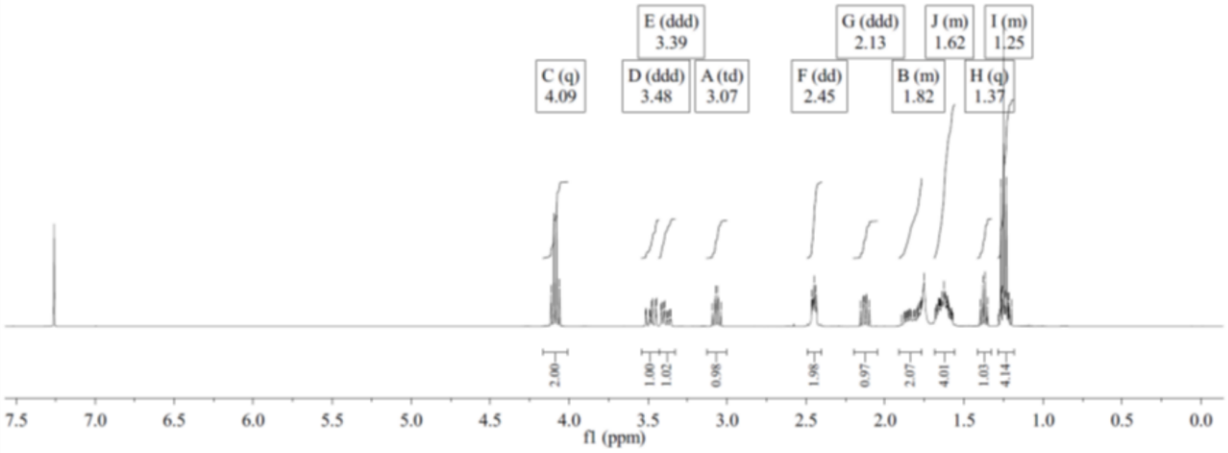

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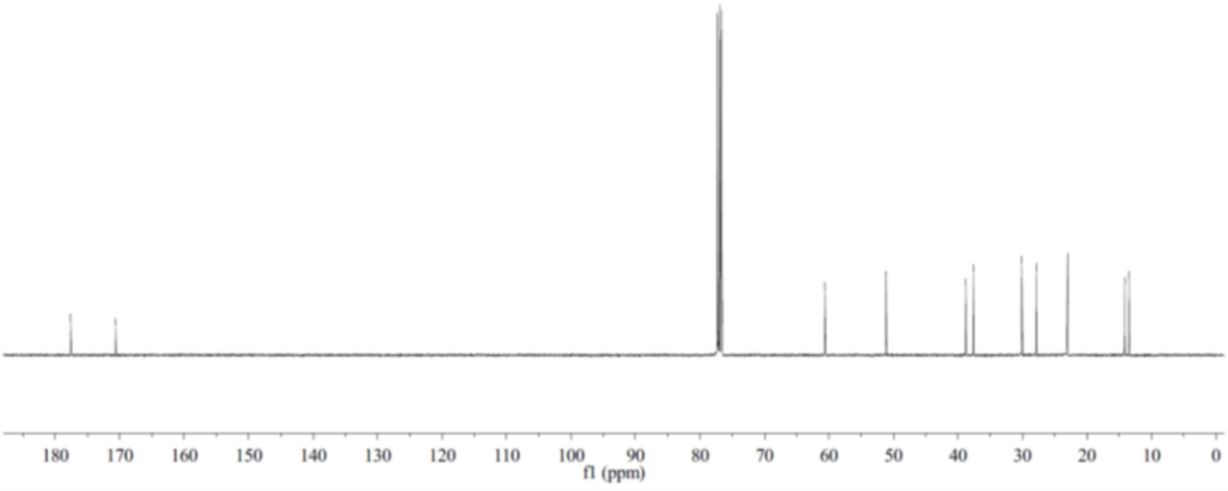

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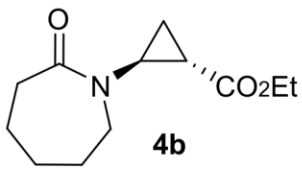

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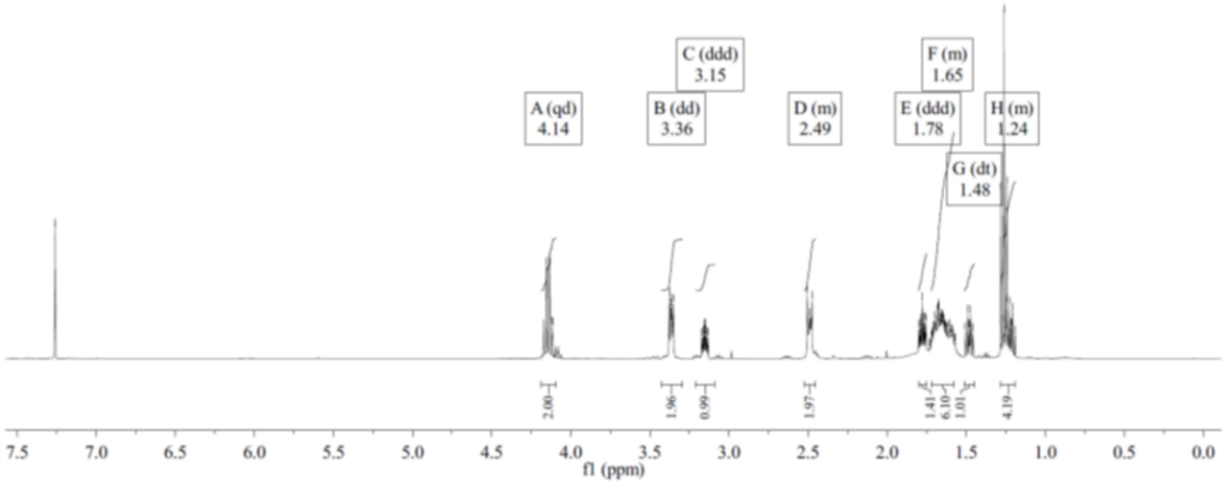

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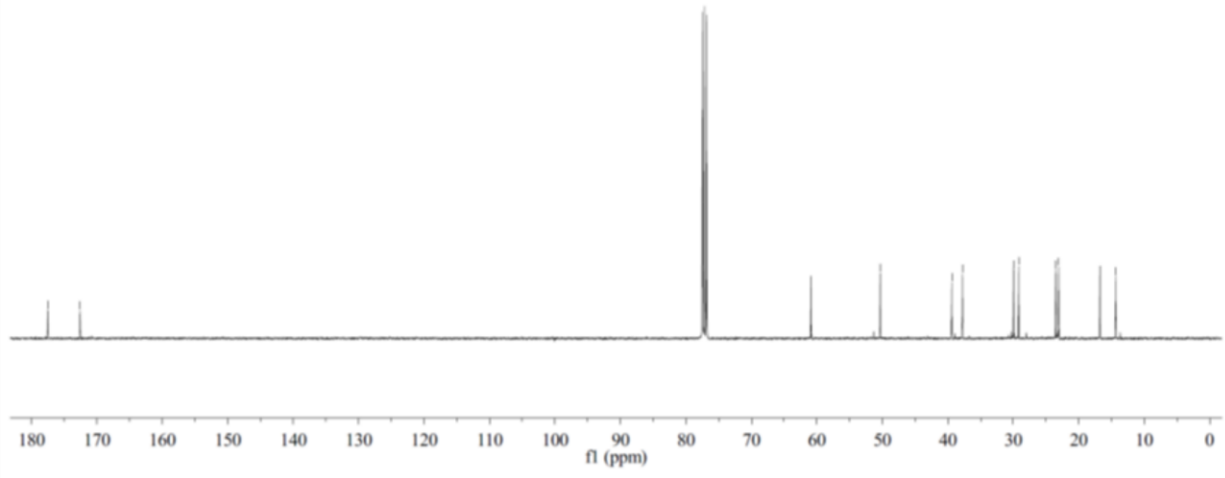

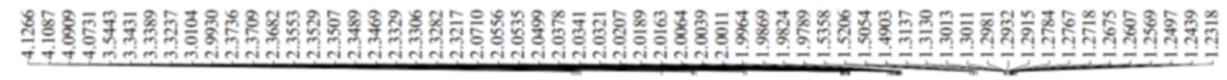

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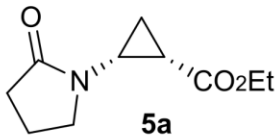
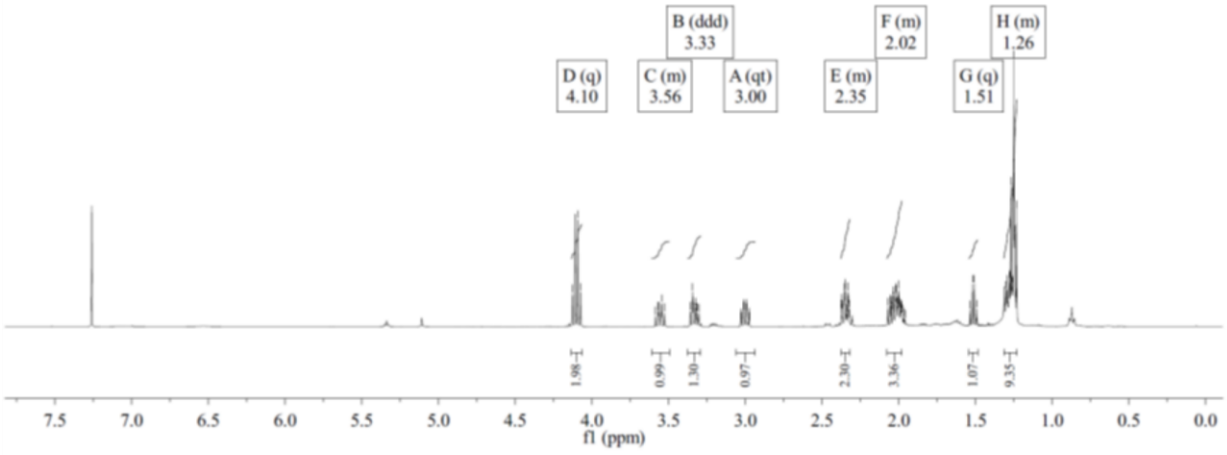


5a



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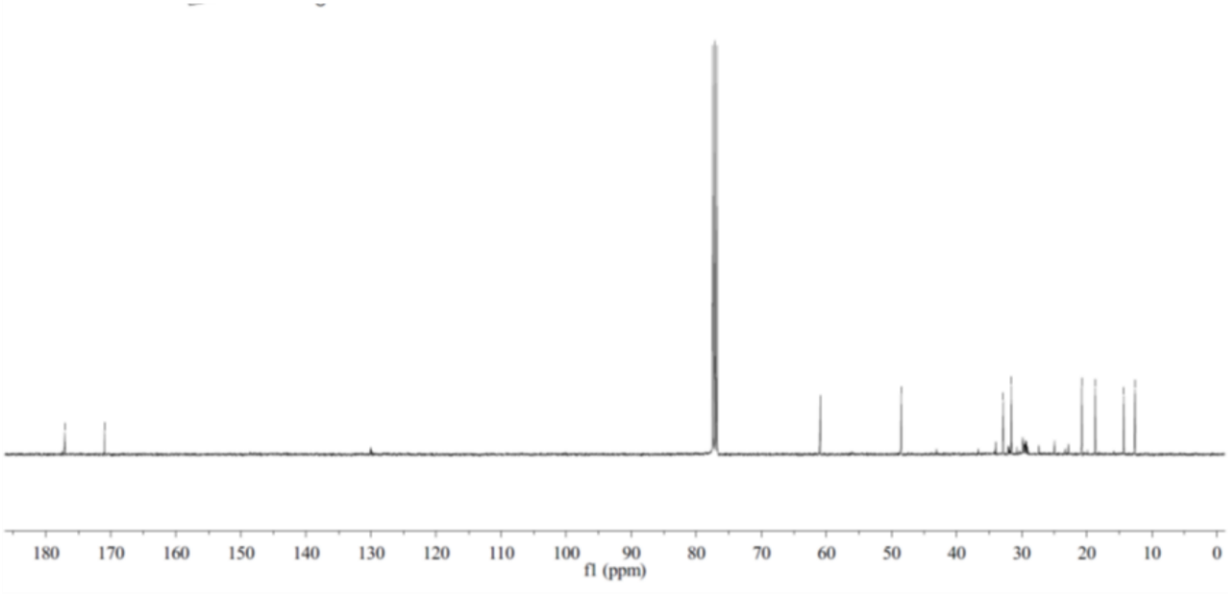

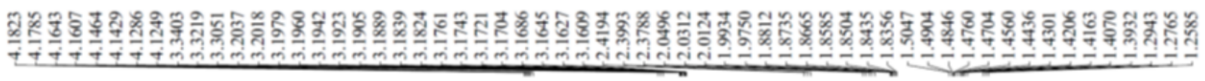

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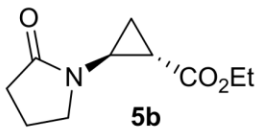
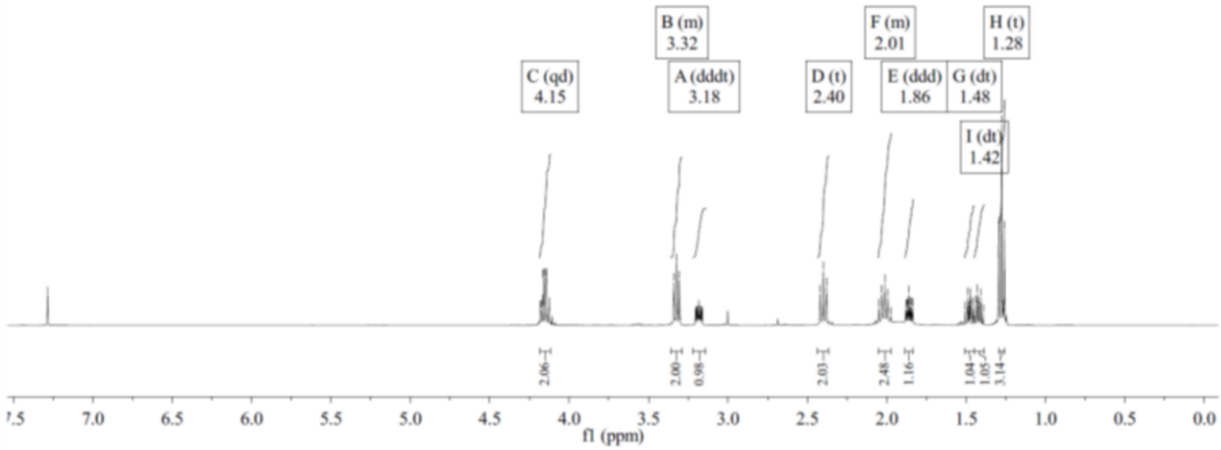


5b



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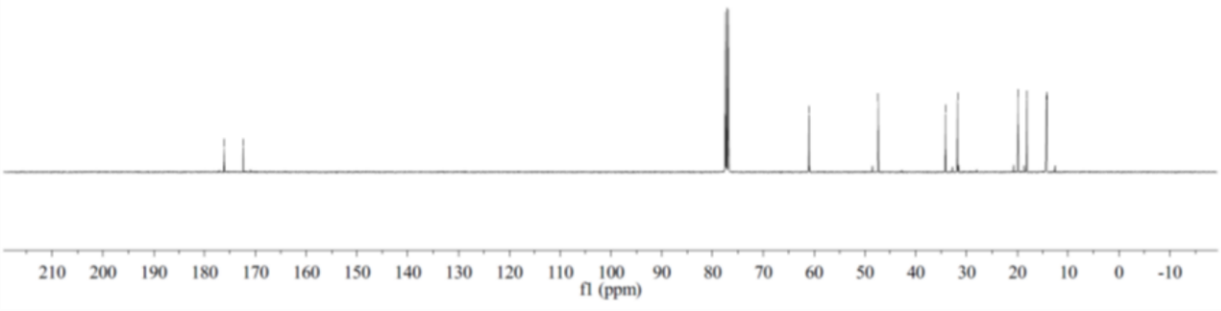

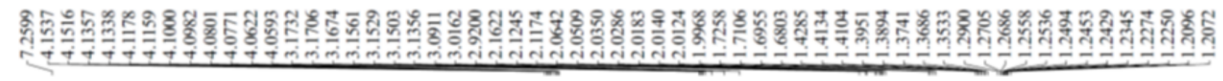

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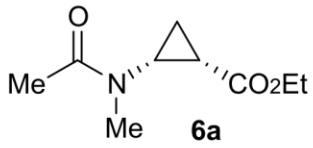
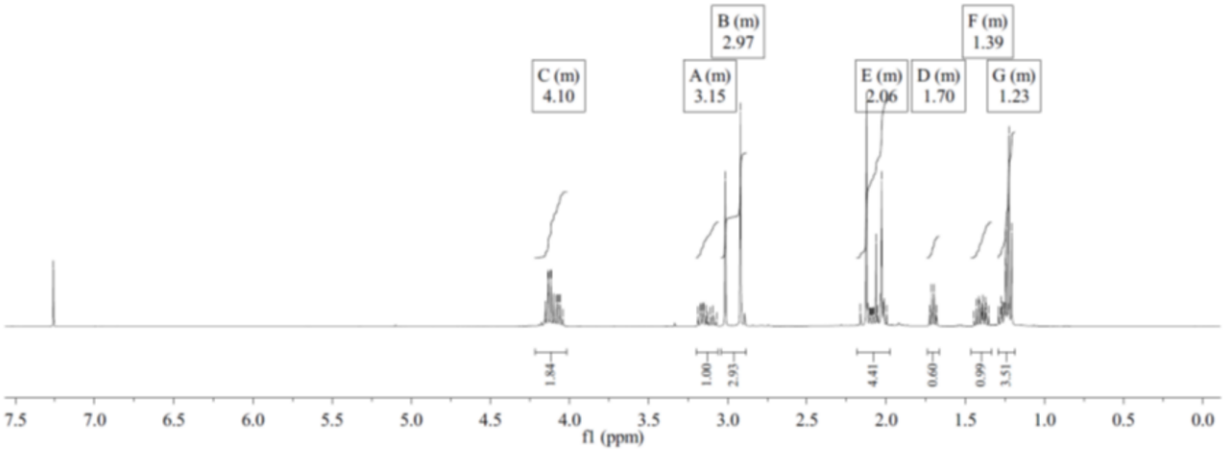


6a



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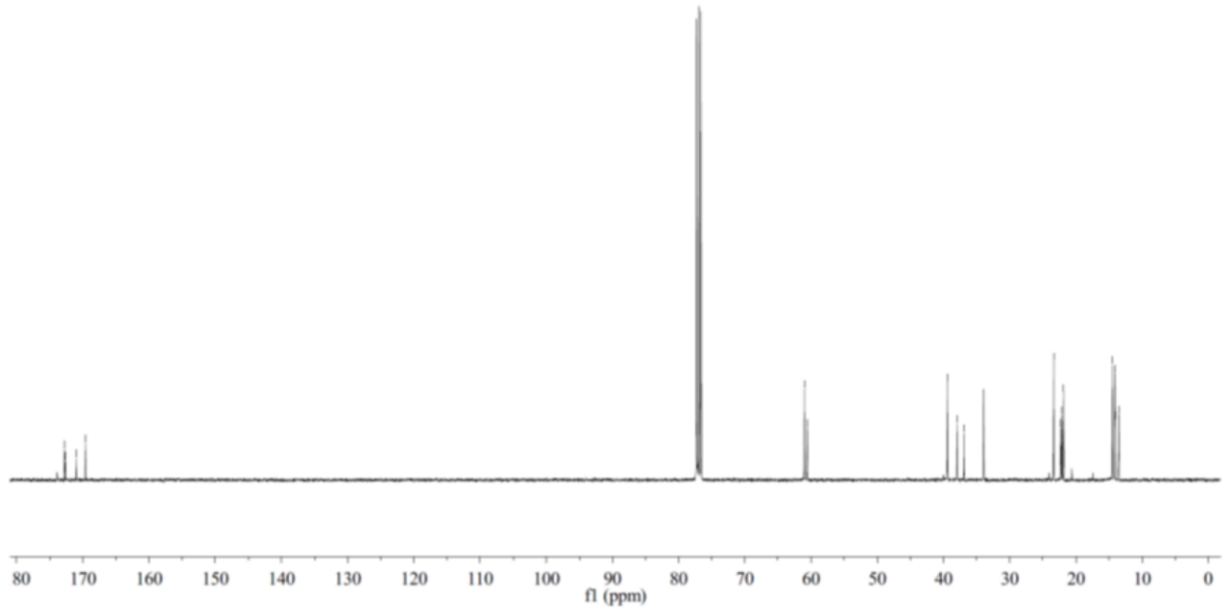

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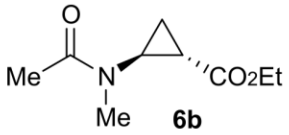

6b

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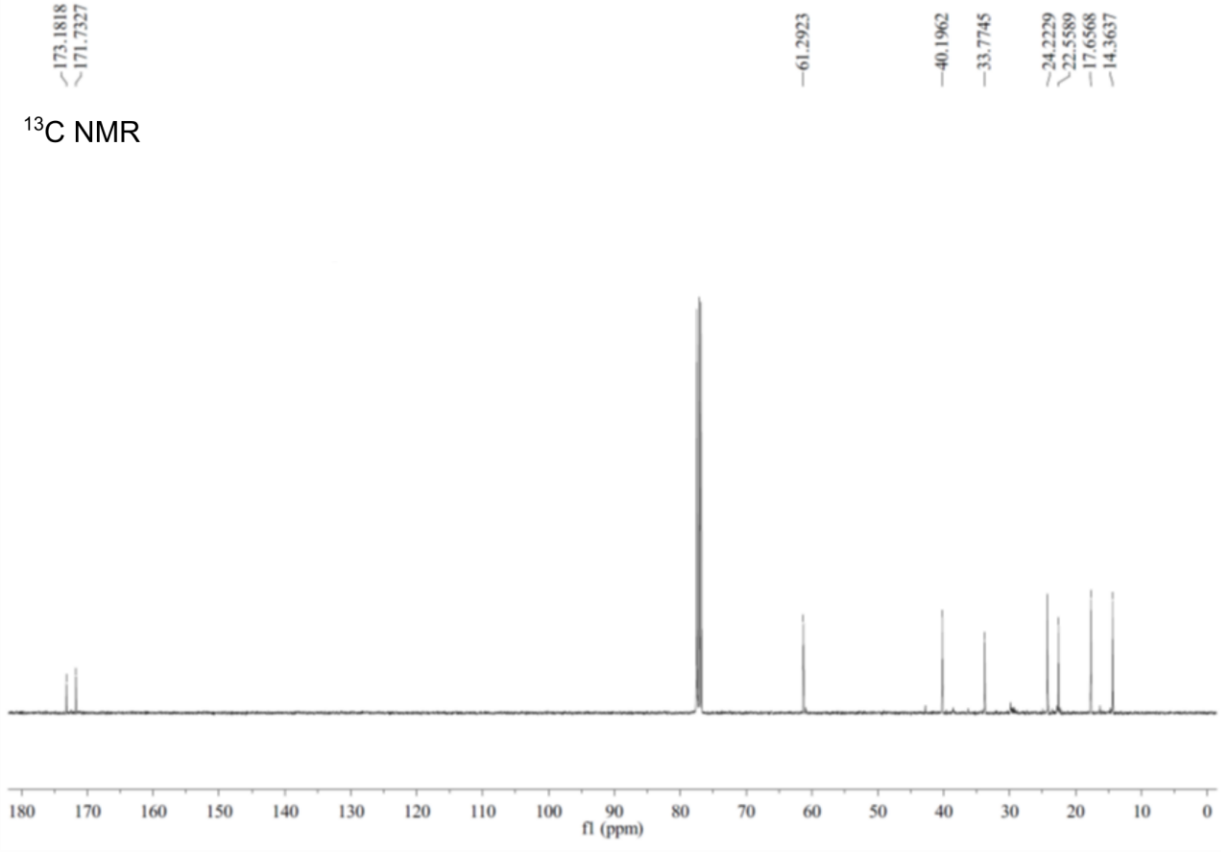

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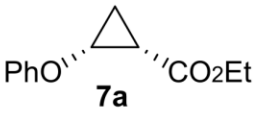

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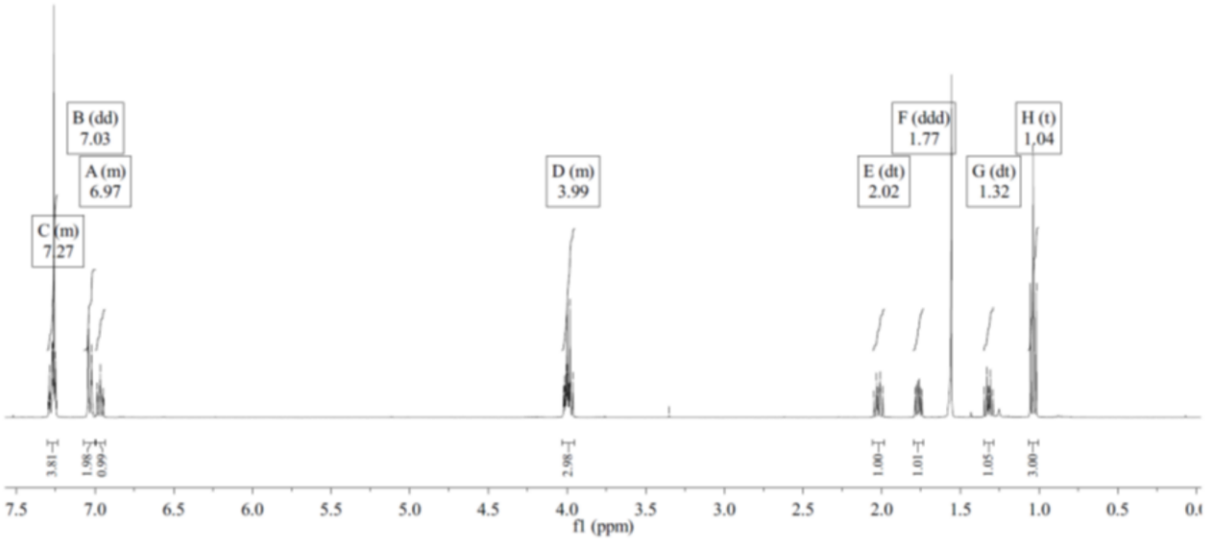

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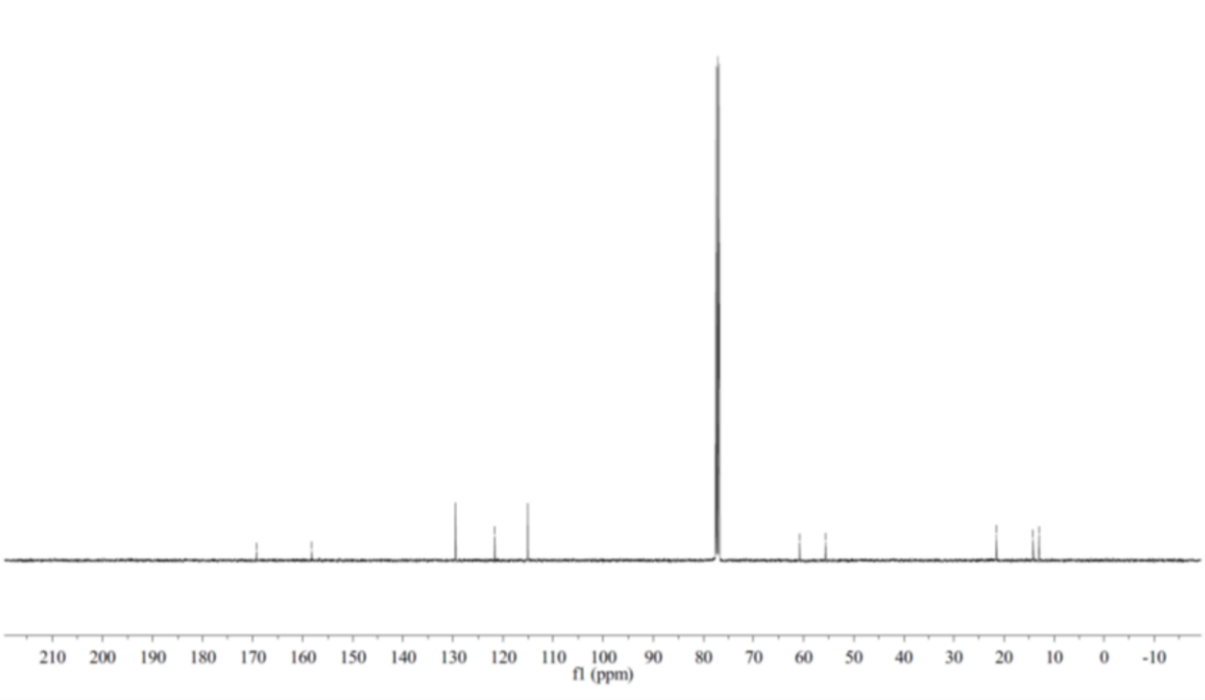

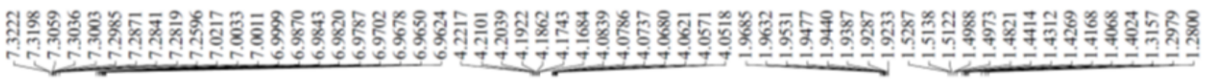

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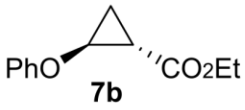

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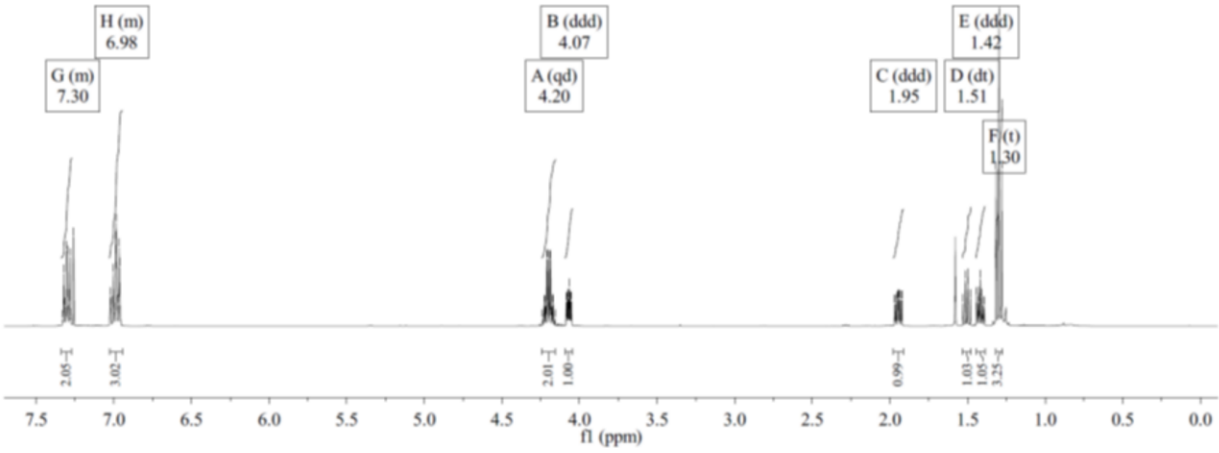

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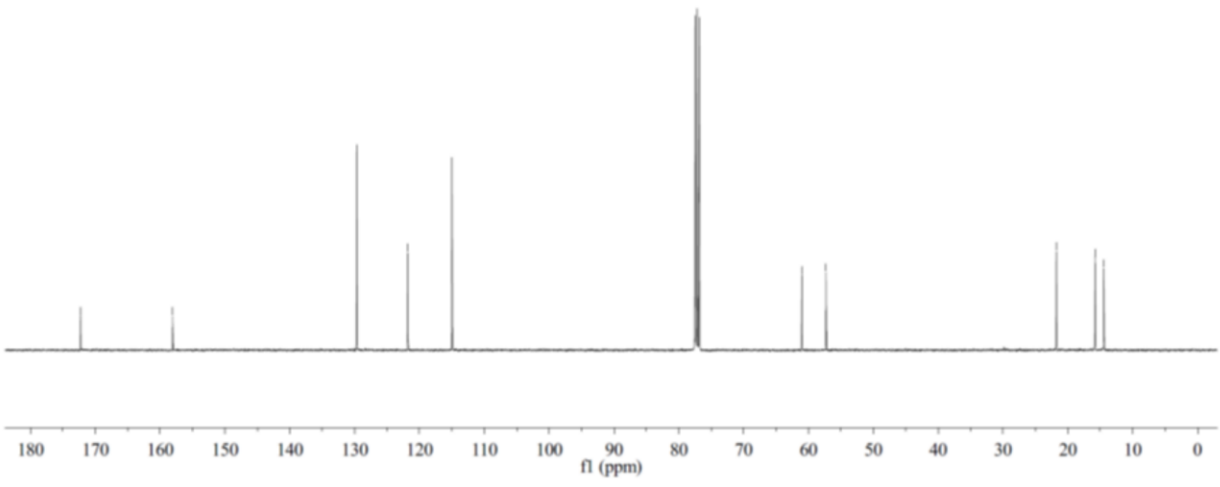

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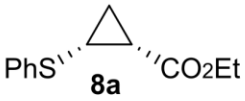

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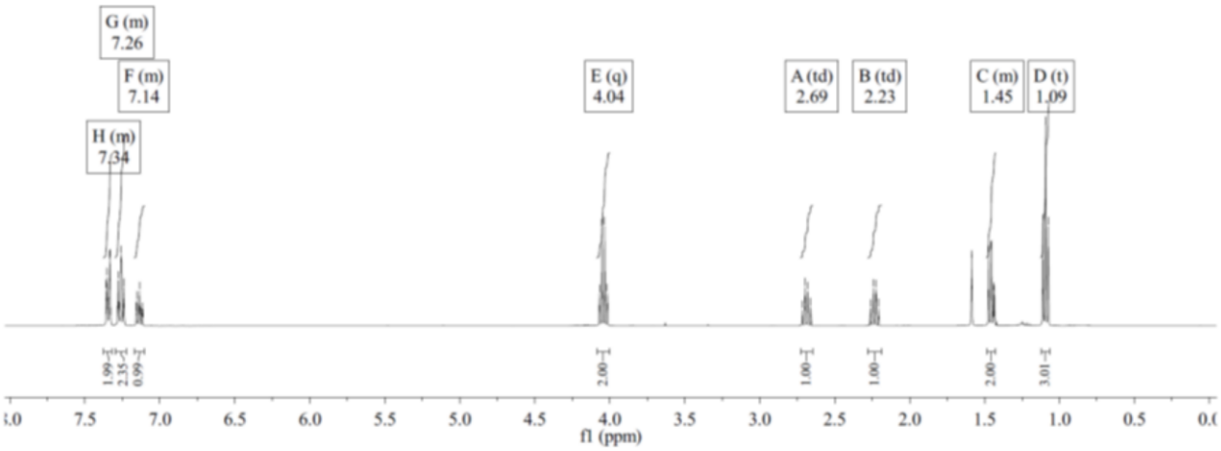

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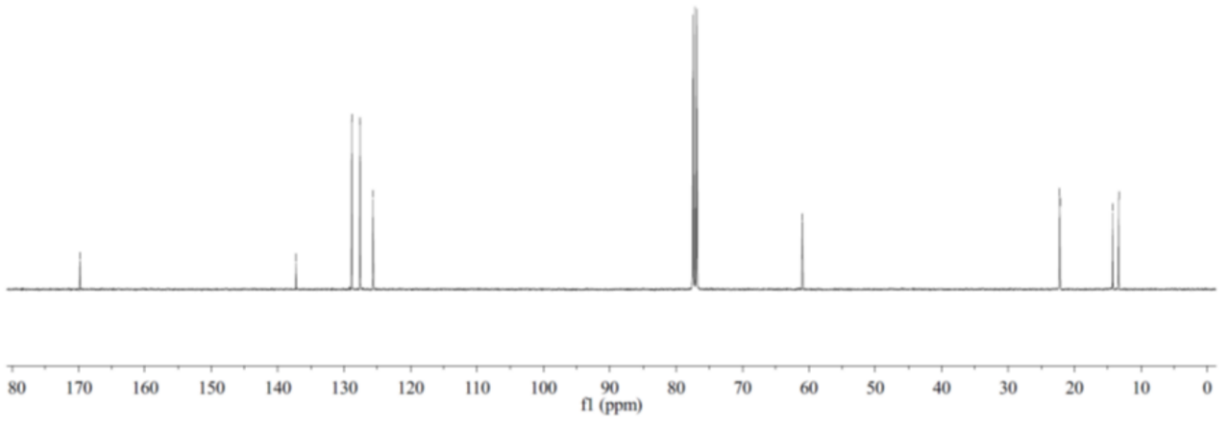

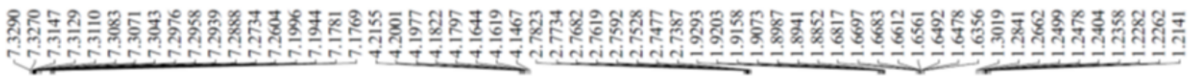

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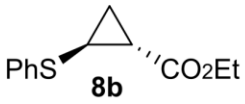

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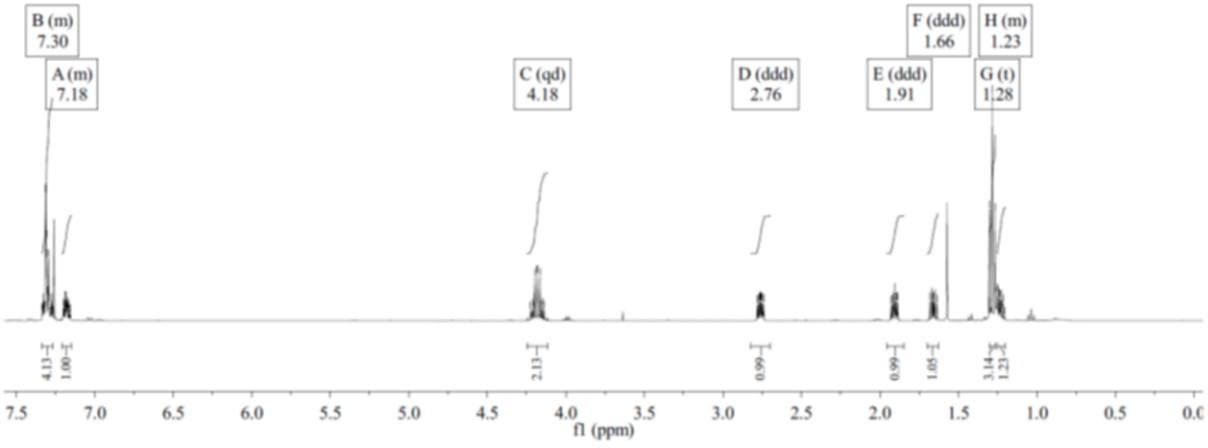

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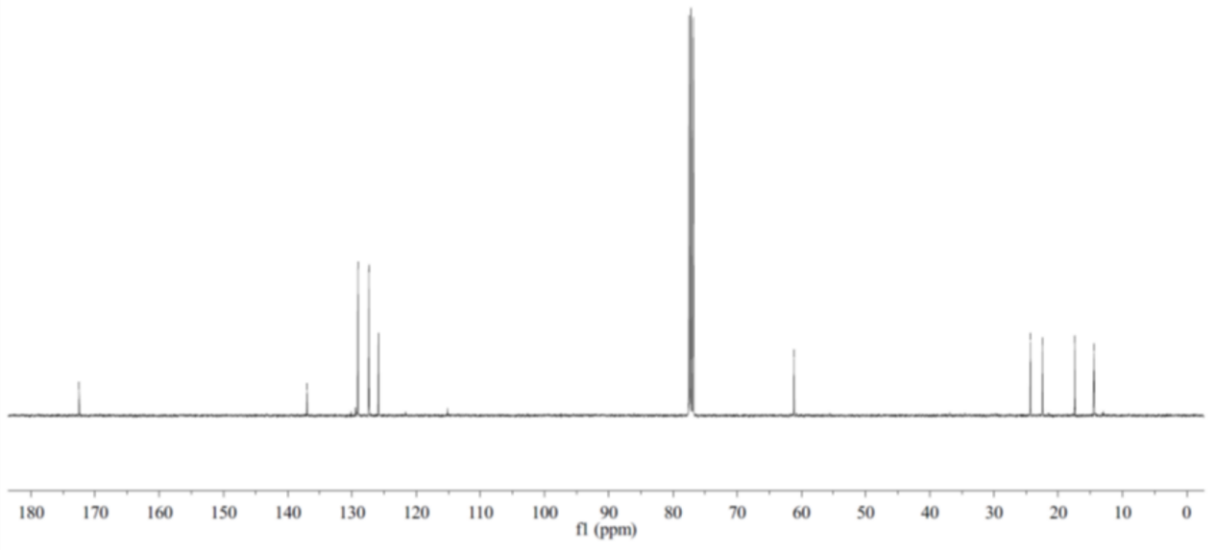

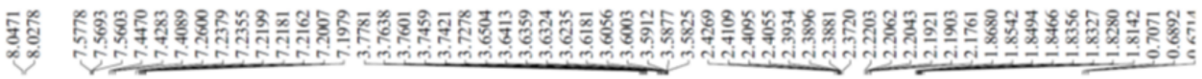

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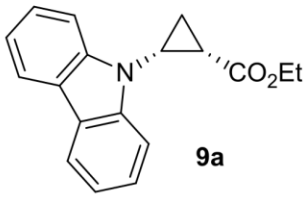

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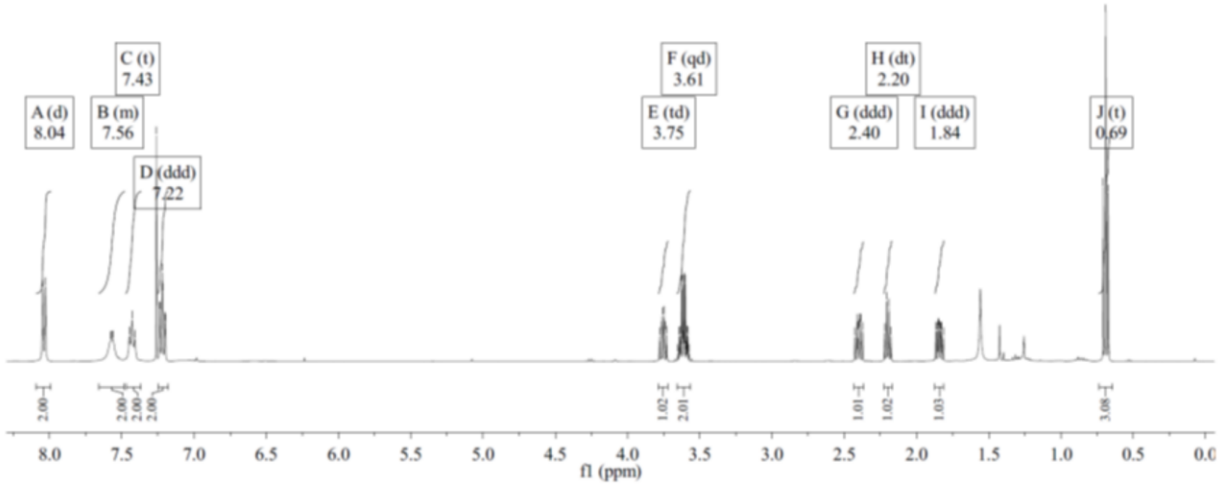
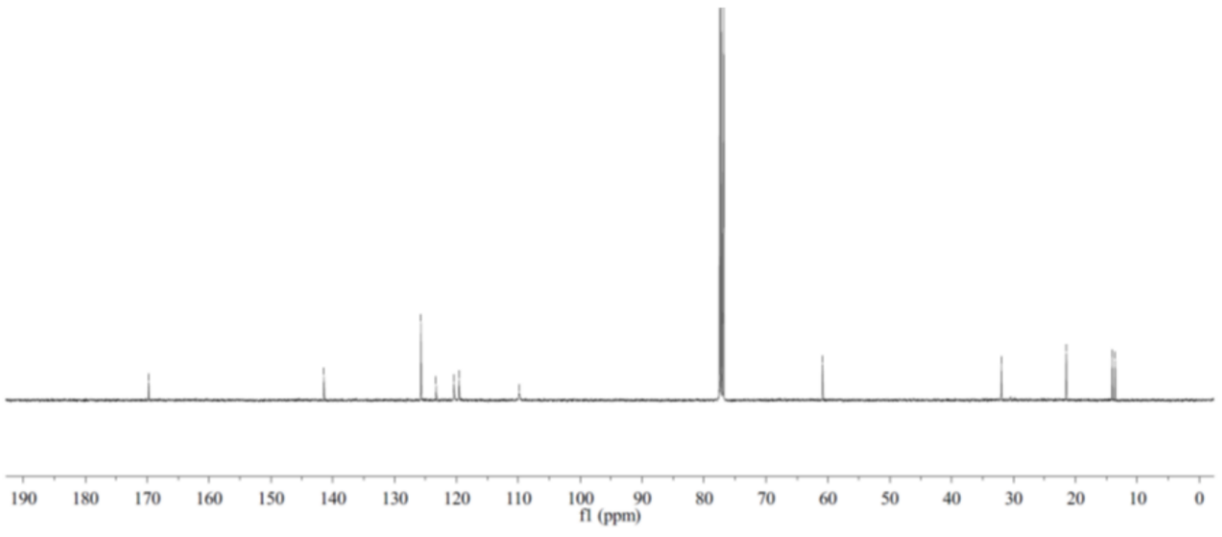
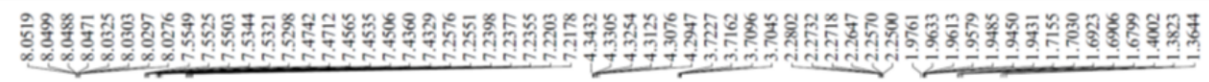


^{13}C NMR





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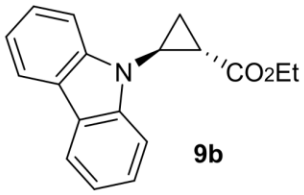

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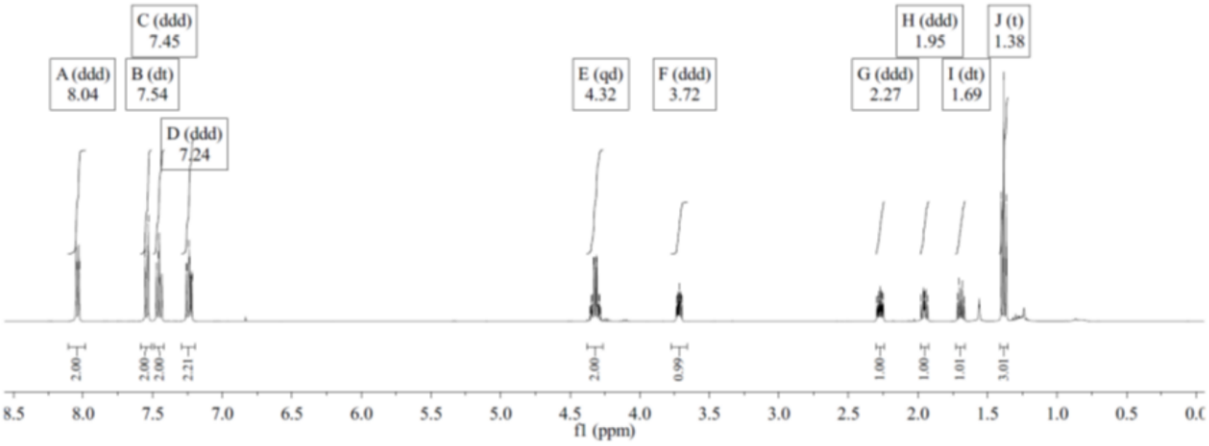

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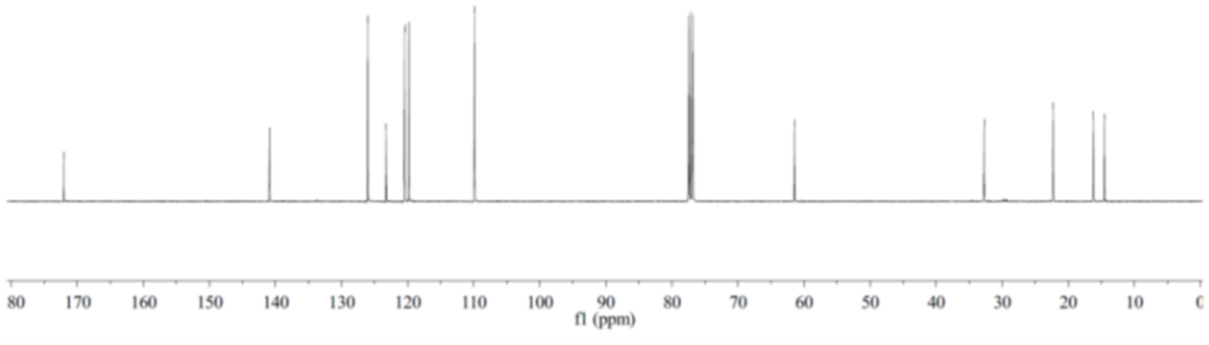

VI. Nucleotide and amino acid sequences of evolved P411 variants

Nucleotide sequence of cytochrome P411-VAC$_{cis}$

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GAATCACGCTTTGATAAAAACTTAAGTCAAGCGTATAAATTTGCACGTGATTTTGCAGGAGACG
GGTTAGTGACAAGCTGGACGCATGAAAAAAATTGGAAAAAAGCGCATAATATCTTACTTCCAAG
CTTTAGTCAGCAGGCAATGAAAGGCTATCATGCGATGATGGTCGATATCGCCGTGCAGCTTGTT
CAAAAGTGGGAGCGTCTAAATGCAGATGAGCATATTGAAGTATCGGAAGACATGACACGTTTAA
CGCTTGATACAATTGGTCTTTGCGGCTTTAACTATCGCTTTAACAGCTTTTACCGAGATCAGCC
TCATCCATTTATTATAAGTATGGTCCGTGCAATTGATGAAGTAATGAACAAGCTGCAGCGAGCA
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ACGACCTAGTAGATAAAATTATTGCAGATCGCAAAGCAAGGGGTGAACAAAGCGATGATTTATT
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Amino acid sequence of cytochrome P411-VAC$_{cis}$

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKEACD
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Nucleotide sequence of cytochrome P411-VAC*trans*

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AGTGAAGCAGACGCTCGCTTATGGCTGCAGCAGCTAGAAGAAAAAGGCCGATACGCAAAAGACG
TGTGGGCTGGGCTCGAGCACCACCACCACCACTGA

Amino acid sequence of cytochrome P411-VAC*trans*

MTIKEMPQPKTFGELKNLPLLNTDKPVQALMKIADELGEIFKFEAPGRVTRYLSSQRLIKEACD
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TTYQKVPAFIDETLAAKGAENIADRGEADASDDFEGTYEEWREHMWSDVAAYFNLDIENSEDNK
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TPLIMVGPGTGVAPFRGFVQARKQLKEQGQSLGEAHLYFGCRSPHEDYLYQEELENAQSEGIIT
LHTAFSRMPNQPKTYVQHVMEQDGKKLIELLDQGAHFYICGDGSQMAPAVEATLMKSYADVHQV
SEADARLWLQQLEEKGRYAKDVWAGLEHHHHHH

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